UNIVERSIDADE FEDERAL DE LAVRAS

NATÁLIA TRAJANO DE OLIVEIRA

ZINC BIOFORTIFICATION IN FOOD-TYPE SOYBEAN CULTIVARS

LAVRAS – MG

2017

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitotecnia, área de concentração em Produção Vegetal, para a obtenção do título de Doutor.

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BIOFORTIFICAÇÃO COM ZINCO EM CULTIVARES DE SOJA TIPO-ALIMENTAÇÃO

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APROVADA em 08 de agosto de 2017.

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Ao Cirano, companheiro de sonhos e aventuras.

Dedico

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Se não houver frutos
valeu a beleza as flores
Se não houver flores
valeu a sombra das folhas
Se não houver folhas
Valeu a intenção da semente.
Henfil

RESUMO

Reduzir os riscos de deficiência de zinco, por meio da diversificação da dieta alimentar e intervenções agrícolas, representa um desafio para o futuro. Com o intuito de verificar a biofortificação com zinco em cultivares de soja tipo-alimentação, três estudos foram conduzidos. No primeiro estudo foi avaliado o efeito de formas de adubação com zinco sobre a biofortificação de cultivares de soja tipo-alimentação. O delineamento experimental utilizado foi o inteiramente casualizado em arranjo fatorial 5x3, com quatro repetições. Foram testadas cinco estratégias de adubação com zinco (controle sem aplicação de zinco, adubação via solo, adubação foliar em estádio vegetativo 4, foliar em vegetativo 8, foliar em reprodutivo 4) em três cultivares de soja (BRS 213, BRSMG 790A e BRS Favorita RR®). No segundo estudo, o objetivo foi verificar o efeito das doses de zinco aplicadas via foliar em cultivares de soja destinados à alimentação humana visando à biofortificação dos grãos. Foi utilizado delineamento inteiramente casualizado com quatro repetições, em esquema fatorial 5x5: cinco cultivares de soja, BRS 010, BRS 213, BRSMG 790A, BRSMG 800A e Favorita RR® combinado com cinco doses foliares de zinco aplicadas no estágio reprodutivo 4 (0, 0,91, 1,82, 2,73 e 6,37 mg L^{-1}). No terceiro, avaliou-se o efeito dos níveis de zinco e ferro em plantas de soja, cultivar BRS 290A, cultivadas em sistema hidropônico dividido em dois experimentos. No primeiro, utilizou-se esquema fatorial 4 x 3: quatro níveis de zinco (2, 10, 20 e 40 μM) e três níveis de ferro (1, 7,7 e 77 μmol L^{-1}) com quatro repetições. No experimento sobre absorção, plantas foram cultivadas em solução modificada contendo três níveis de ferro (1, 7,7 e 77,7 μmol L^{-1}) combinados com a alta concentração de zinco (40 μM). As plantas foram colhidas em diferentes tempos para estudo da absorção (1, 2, 4, 6, 10, 16, 22, 24, 32 e 48 horas). A adubação com zinco via foliar em estádio de desenvolvimento reprodutivo 4 promove a maior biofortificação com zinco nos grãos de soja quando em condições de baixa disponibilidade deste nutriente no solo. A cultivar BRSMG 790A se destacou quanto ao acúmulo de zinco apresentando maior incremento de zinco no grão e eficiência no uso do nutriente. O aumento nas doses de zinco aplicadas via foliar promove respostas crescentes no conteúdo desse nutriente em grãos soja. Diferentes níveis de Zn e Fe combinados afetam a absorção e o estado mineral, bem como o crescimento de plantas de soja, promovendo diferentes fenótipos.

Palavras-chave: Adubação foliar. Composição mineral. *Glycine max* (L.) Merrill. Micronutrientes.

ABSTRACT

Reducing risks of zinc deficiency by diversifying diet and agricultural interventions represents a challenge for the future. To verify zinc biofortification in food-type soybean cultivars, we carried out three studies. In the first one, we assessed the effects of fertilization with zinc on the biofortification of food-type soybean cultivars using the fully randomized design with a 5x3 factorial arrangement and four repetitions. We tested five strategies of fertilization with zinc (control without zinc, soil fertilization, fertilization of leaves at vegetative stage 4, leaves at vegetative stage 8, leaves at reproductive stage 4) on three soybean cultivars (BRS 213, BRSMG 790A and BRS Favorita RR®). The second study aimed at verifying the effect of doses of zinc applied on the leaves of food-type soybean cultivars in order to fortify the grains. We used the fully randomized design with four repetitions and a 5x5 factorial arrangement: five soybean cultivars (BRS 010, BRS 213, BRSMG 790A, BRSMG 800A and Favorita RR®) combined with five doses of zinc applied to leaves at reproductive stage 4 (0, 0.91, 1.81, 2.73 and 6.37 mg L^{-1}). In the third study, we evaluated the effect of zinc and iron levels on soybean plants (cultivar BRS 290A), cultivated in a hydroponic system divided into two experiments. We used a 4x3 factorial arrangement in the first one: four levels of zinc (2, 10, 20 and 40 μM) and three levels of iron (1, 7.7, and 77 μmol L^{-1}) with four repetitions. In the experiment on absorption, the plants were cultivated in a modified solution with three levels of iron (1, 7.7 and 77.7 μmol L^{-1}) combined with a high concentration of zinc (40 μM). The plants were harvested at different periods to study absorption (1, 2, 4, 6, 10, 16, 22, 24, 32 and 48 hours). The fertilization with zinc through leaves at stage 4 of reproductive development promotes greater biofortification of soybean grains, when the soil lacks this nutrient. Cultivar BRSMG 790A was the best and most effective at accumulating zinc and implementing it to the grains. Increasing the doses of zinc applied to the leaves promotes fast responses to the content of this nutrient in soybean grains. Different levels of Zn and Fe combined affect the absorption and the mineral state, as well as the growth of soybean plants, thus creating different phenotypes.

Keywords: Leaf fertilization. Mineral composition. *Glycine max* (L.) Merrill. Micronutrients.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO GERAL

Nos últimos 10 anos, 167 milhões de pessoas deixaram de passar fome, no entanto cerca de 795 milhões ainda sofrem com esse problema. Tão grave como a falta de alimento é a carência não explícita de um ou mais micronutrientes no organismo, a “fome oculta”. Nessa condição, os estoques de vitaminas e minerais diminuem silenciosamente, sem apresentar sinais nem sintomas, os quais, só ficam evidentes, quando o estágio mais grave da deficiência está instalado. Aproximadamente um terço da população sofre com deficiências de vitaminas, particularmente A e C, e de minerais como zinco (Zn), iodo (I) e ferro (Fe) (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS - FAO, 2015).

Nesse âmbito, a biofortificação de plantas é uma alternativa para complementar os programas de intervenção nutricional existentes, reduzindo os problemas de deficiências (RIOS et al., 2008). São duas as principais estratégias utilizadas: a biofortificação genética pela seleção de genótipos capazes de maior acúmulo do nutriente nos tecidos, e a biofortificação agronômica, por meio do enriquecimento pela adição do nutriente pela adubação (ZHU et al., 2009). Segundo a FAO (2015) a biofortificação é uma maneira de alcançar os consumidores que têm dificuldade em acessar dietas ricas em micronutrientes, suplementos alimentares ou alimentos processados fortificados convencionalmente.

Além de importante para saúde humana, o Zn também desempenha papel fundamental nas plantas. Dentre as principais funções do Zn nas plantas destacam-se: ativador ou componente estrutural de enzimas; participação na fotossíntese de plantas C4, por meio da enzima carboxilase pirúvica; necessário para a produção de triptofano, aminoácido precursor do ácido indol acético, hormônio vegetal de crescimento; está envolvido no metabolismo do nitrogênio e é necessário para manutenção da integridade das biomembranas (MALAVOLTA, 2006).

Regiões com solos deficientes em Zn são também regiões onde a deficiência de Zn em seres humanos é difundida. Assim, tornam-se relevantes pesquisas com foco no desenvolvimento de métodos de aplicação de Zn, para promover a absorção e maximizar a acumulação de Zn em órgãos comestíveis da planta, tornando disponível à população alimentos com maiores teores desse nutriente. Dessa forma, a biofortificação pode ser uma estratégia útil e eficaz na resolução de problemas de saúde global (CAKMAK, 2008).

Atualmente, a soja [*Glycine max* (L.) Merrill] se destaca como uma das culturas mais importantes no fornecimento proteína vegetal. Adicionalmente, há a tendência de aumento no consumo de alimentos que garantam um bom aporte nutricional com benefícios à saúde. Assim, a demanda pelo uso direto da soja na alimentação humana tem aumentado. Trata-se de alimento nutricionalmente rico, servindo para saciar a fome e nutrir o organismo, colaborando para a manutenção da saúde da população em geral (PENHA et al., 2007).

Esse cenário, estimulou o desenvolvimento de novas cultivares tipo-alimentação, para usos e nichos diferenciados de mercado, para isso foi necessário melhorar a composição nutricional e eliminar ou diminuir a presença das lipoxigenases, enzimas que promovem a oxidação e instabilidade de ácido graxos presentes na soja. Estes são responsáveis pelo sabor desagradável, “off flavor”, fator limitante ao consumo de soja (ESTEVES et al., 2010).

Pesquisa com biofortificação agronômica visando a aumentar os teores de Zn, selênio e iodo por meio da adubação via solo, evidenciou a capacidade da soja em absorver Zn nos grãos, apresentando teores entre 57 e 59 mg kg^{-1}, superior aos teores encontrado no trigo (15 a 16 mg kg^{-1}), no milho (16 a18 mg kg^{-1}), na batata (13 a 15 mg kg^{-1}), na canola (28 a 36 mg kg^{-1}) e no repolho (20 a 61 mg kg^{-1}) sob as mesmas condições experimentais (MAO et al., 2014).

Estudos com Zn sempre foram direcionados para o manejo nutricional das plantas, com pouco enfoque na possibilidade da biofortificação de plantas para a alimentação humana. No país, poucos estudos têm sido realizados com a soja para alimentação humana. Assim, estudos abrangendo a temática da biofortificação, visando ao aumento dos teores encontrados nos grãos por meio da apropriada aplicação de Zn ou seleção de materiais com boa capacidade de acúmulo devem ser fomentados.

2 REFERENCIAL TEÓRICO

2.1 Importância do Zn para os organismos

O zinco é um nutriente essencial para todos os organismos vivos. Em humanos, é essencial para a atividade de mais de 300 enzimas, relacionado a processos mitóticos, síntese de DNA e proteínas, expressão e ativação gênica, o que enfatiza sua importância durante os períodos de gestação (CAULFIELD et al., 1998; OSENDARP; WEST; BLACK, 2003).

A deficiência de Zn está diretamente relacionada à entrada, absorção inadequada e à presença de inibidores na dieta. Assim, as exigências em zinco são maiores naquelas populações em que os produtos de origem animal, fontes de zinco de maior biodisponibilidade, são limitados (LÖNNERDAL, 2000).

A deficiência de Zn afeta, aproximadamente, um terço da população mundial (INTERNATIONAL FOOD POLICY RESEARCH INSTITUTE - IFPRI, 2016). Essa deficiência está envolvida com quadros de infecções respiratórias e diarreias. Em geral, 1,4% das mortes são atribuídas à carência de zinco: 1,4% nos homens e 1,5% nas mulheres sendo considerado como um problema de saúde pública (WORLD HEALTH ORGANIZATION - WHO, 2008). Em razão da frequente deficiência em dietas humanas têm se estimulado o uso de estratégias para aumentar seu conteúdo nos cultivos alimentares (HIRSCHI, 2009).

Nas plantas, esse nutriente também desempenha funções importantes como: componente integral de milhares de proteínas; múltiplos papéis fisiológicos em uma variedade de componentes de processos biológicos; fator catalítico estrutural e o mediador de sinalização (JOY et al., 2015; KAMBE et al., 2015).

A atividade do Zn é efetiva para determinados processos relevantes na homeostase fisiológica e nutricional da planta, atuando como ativador ou componente estrutural de enzimas; participa da fotossíntese nas plantas C4, através da enzima carboxilase pirúvica; é necessário para a produção de triptofano, aminoácido precursor do ácido indol acético, hormônio vegetal de crescimento, está envolvido no metabolismo do nitrogênio, das proteínas e lipídeos, além de ser necessário para a manutenção da integridade das biomembranas (MALAVOLTA, 2006; MARSCHNER, 1995).

Por estar envolvido em uma série de processos fisiológicos nas plantas, pequenas deficiências podem causar danos no crescimento, rendimento e no teor de Zn em partes comestíveis (KUMAR; MEENA; SINGH, 2016). A absorção nas plantas é fortemente influenciada por fatores ambientais como altas temperaturas, seca, níveis de outros nutrientes e metais pesados na rizosfera e populações microbianas do solo influenciam a absorção de Zn nas plantas (GUPTA; RAM; KUMAR, 2016).

Regiões com solos deficientes em Zn coincidem com regiões onde a deficiência de Zn em seres humanos tem alta prevalência. Assim, tornam-se relevantes pesquisas com foco no desenvolvimento de métodos de aplicação de Zn eficientes que que promovam a absorção de Zn e maximizem a acumulação em partes comestíveis, tornando disponível à população alimentos com maiores teores desse nutriente (CAKMAK, 2008).

O Zn é geralmente absorvido pelas plantas na forma de Zn^{2+}, via radicular ou foliar. A partir da solução do solo, sua absorção pode ser realizada por fluxo de massa, difusão ou interceptação radicular, dependendo das concentrações iônicas na superfície radicular, da necessidade das plantas e da capacidade de absorção de raízes (FERNANDES, 2006; MARSCHNER, 1995).

A absorção de Zn também é dependente da presença de proteínas de transporte e agentes quelantes (ácidos orgânicos, fitosideróforos) cuja expressão diferencial é responsável pelas diferenças interespecíficas e intraespecíficas nas plantas. O mecanismo de absorção de Zn nas plantas é rigorosamente controlado. O Zn é mais absorvido na parte aérea do que nas raízes (folhas > caule > flor / fruto). Nos tecidos, a absorção máxima de Zn é em tecidos vasculares e parênquima cortical independente da raiz ou parte aérea, com maior acúmulo no mesofilo. Nas células, a concentração é maior no vacuolo que no citosol (GUPTA; RAM; KUMAR, 2016).

Como o Zn não pode se difundir por meio da membrana, utiliza transportadores, que facilitam a circulação do nutriente nas células e seu transporte para fora das organelas intracelulares. As principais famílias de transportadores envolvidas são ZIP (ZRT, IRT-like Protein), IRT (Iron-regulated transporter), NRAMP (Natural resistance-associated macrophage protein), CDF (Cation diffusion facilitator) e ZRT (Zinc regulation transporter) (KUMAR; MEENA; SINGH, 2016).

2.2 Zinco na agricultura

Dada a sua importância, o Zn deve estar disponível em quantidades adequadas, uma vez que fatores associados ao solo como níveis elevados de carbonato de cálcio, óxidos metálicos, pH, matéria orgânica, umidade do solo, quantidades elevadas de fosfato podem reduzir a solubilidade do Zn (CAKMAK, 2010).

Deficiências de Zn vêm ocorrendo em uma ampla variedade de solos em todo o mundo, sendo esse agravado com o cultivo intensivo no solo, perdas de solo por erosão, altos valores de pH, provocados por calagem excessiva e redução dos teores de matéria orgânica (ALMEIDA JÚNIOR et al., 2007; FAGERIA; COSTA, 2000).

No Brasil, a deficiência de Zn ocorre principalmente pelos baixos teores naturais nos solos, além da utilização de quantidades relativamente elevadas de calcário para correção da acidez e pelo uso de fertilizantes fosfatados, uma vez que há antagonismo entre P e Zn (FAGERIA, 2012).

O modo de aplicação do Zn é fundamental para garantir maior absorção pela planta (CORREIA et al., 2008). Podem-se destacar os seguintes métodos de aplicação: via solo (localizado ou incorporado), foliar ou via sementes. Entretanto, a aplicação de pequenas doses tem sido suficiente para corrigir deficiências e promover um efeito residual (BARBOSA FILHO; FAGERIA; CARVALHO, 1982; RITCHEY et al., 1986).

De acordo com Orioli Júnior et al. (2008), o modo de aplicação de zinco no solo de forma localizada proporciona maiores concentrações do micronutriente disponível, embora não tenha influenciado o crescimento inicial das plantas de trigo e a aplicação foliar proporciona o maior acúmulo do nutriente na massa seca da parte aérea, em decorrência baixa mobilidade desse nutriente no floema.

Resposta da soja à adubação com zinco em solo com teores acima do nível crítico mostram que a variação da exportação de Zn pela cultura varia em função das produtividades obtidas. Desse modo, fica evidente que a produtividade tem impacto importante na dinâmica e nos estoques de Zn no sistema solo‑planta, fazendo com que a restituição do nutriente exportado seja constantemente considerada nos programas de adubação (INOCÊNCIO et al., 2012).

2.3 Biofortificação de alimentos com Zn

O envolvimento da agricultura com a nutrição e a saúde humana pode oferecer soluções eficientes, sustentáveis e econômicas para a diminuição de problemas de saúde, relacionados à deficiência de micronutrientes (MELASH; MENGISTU; ABERRA, 2016).

Há indícios de que regiões com solos deficientes em Zn sejam também regiões onde a deficiência de Zn em seres humanos é difundida (CAKMAK, 2008). Cerca de metade dos solos do mundo são deficientes em Zn. Dessa forma, o cultivo de alimentos nesses solos faz com que a concentração de Zn nos alimentos produzidos sejam ainda menores (DAS; GREEN, 2013). Esse problema ainda pode ser agravado com o uso intensivo do solo (ALMEIDA JÚNIOR et al., 2007).

Plantas com deficiência de Zn apresentam baixa concentração do nutriente em seus tecidos. Portanto, além da redução nos rendimentos, os produtos comestíveis provenientes dessas plantas terão menor contribuição para o conteúdo de Zn na dieta humana. Se a concentração de Zn em alimentos básicos é aumentada, grandes serão os benefícios sobre consumo de Zn na dieta humana (ALLOWAY, 2008).

Vários programas de pesquisa associados à biofortificação com Zn em cereais estão em andamento em escala global visando à diminuição dos quadros de desnutrição humana (GUPTA; RAM; KUMAR, 2016).

Programas como “HarvestPlus” iniciaram pesquisas com biofortificação para a produção de cultivos capazes de aumentar tanto a quantidade como a biodisponibilidade de nutrientes em dietas humanas (WHITE; BROADLEY, 2009). Um dos seguimentos desse programa é o “HarvestZinc”, que visa a incrementar as concentrações de Zn em culturas como o trigo e o arroz, baseado em informações obtidas por pesquisas desenvolvidas em vários países, cujos primeiros resultados revelam a viabilidade da estratégia e seu vasto potencial na redução da deficiência de Zn e promovendo impacto positivo na saúde humana (CAKMAK, 2012). Neste programa foram estabelecidas metas de aumento nos teores de Zn baseados nas médias normalmente encontradas, como por exemplo, aumentar o teor de Zn de 16 para 28 mg kg^{-1} no arroz; de 25 para 37 mg kg^{-1} no trigo; de 47 para 77 mg kg^{-1} no milheto, entre outras espécies amplamente consumidas.

A biofortificação é o processo de melhoria do teor de nutrientes em cultivos alimentares, visando a promover o fornecimento de alimentos enriquecidos nutricionalmente. Busca-se reduzir os quadros de desnutrições graves nas populações sem acesso a alimentos e suplementos fortificados (FAO, 2013; FAO, 2015).

São duas as principais estratégias utilizadas visando à biofortificação de plantas: a biofortificação agronômica, pela aplicação de fertilizantes fonte de nutrientes importantes na nutrição humana e a biofortificação genética, por meio do melhoramento de plantas (CAKMAK, 2008; STEIN, 2010; ZHU et al., 2009).

A biofortificação agronômica de cultivos alimentares com nutrientes importantes para a saúde humana tem sido considerada uma estratégia de saúde pública, visando à redução de quadros de deficiência nutricional (JOY et al., 2015). Essa estratégia é importante no enriquecimento de grãos em curto prazo, pela aplicação de fertilizantes fonte Zn, principalmente em solos com severa deficiência desse nutriente (VELU et al., 2014).

A eficiência da biofortificação no aumento da concentração de Zn nos cultivos é altamente influenciada pelo método e época de aplicação da adubação com Zn em função do estádio de desenvolvimento da cultura (MELASH; MENGISTU; ABERRA, 2016). O modo de aplicação é fundamental para garantir maior absorção do nutriente pela planta (CORREIA et al., 2008). O custo/benefício do Zn aplicado via foliar parece ser equivalente à fortificação de farinhas básicas, etapa comum no processo de industrialização (JOY et al., 2015).

Pesquisas com foco no desenvolvimento de métodos de aplicação de Zn mais eficientes para promover a absorção de Zn e maximizar o acúmulo em partes comestíveis são relevantes. Se houvesse maior biodisponibilidade de Zn no grão, derivado da aplicação foliar em comparação a outros métodos, a biofortificação agronômica se tornaria uma estratégia muito atraente, útil e eficaz na solução de problemas de saúde global relacionados com a deficiência de Zn (CAKMAK, 2008).

A biofortificação genética, por meio do melhoramento vegetal pode proporcionar uma solução mais sustentável e rentável em longo prazo no fornecimento de minerais para a população. Essa estratégia envolve tanto o melhoramento tradicional com a exploração da variação natural, visando à seleção de genótipos eficientes no acúmulo de nutrientes, quanto ao uso da biotecnologia, pelo estudo do controle genético e mecanismos fisiológicos moleculares que contribuam para alta acumulação de nutrientes em partes comestíveis das plantas (CAKMAK, 2008; WHITE; BROADLEY, 2009).

Estudos com transgenia demonstram claramente a expressão de proteínas de transporte e ligantes como a nicotianamida e ácidos orgânicos na aquisição de Zn. Além disso, a elucidação de genes e mensageiros atuantes na regulação da absorção, acumulação e translocação de Zn representam uma via promissora para o entendimento do mecanismo de absorção do nutriente nas plantas. Entender os mecanismos de absorção, translocação e homeostase do Zn deve ser objetivo dos programas de biofortificação, a fim de enriquecer o tecido alvo com esse micronutriente (GUPTA; RAM; KUMAR, 2016).

Compreender a dinâmica do fornecimento do nutriente até os grãos em crescimento permitirá o desenvolvimento de cultivares melhoradas que respondam favoravelmente na manutenção da qualidade e da produção de grãos em ambientes adversos (WATERS; SANKARAN, 2011).

É notório que a biofortificação genética e agronômica são complementares e oferecem uma solução sustentável para a redução de problemas de desnutrição relacionados com micronutrientes. Em longo prazo, a biofortificação agronômica é uma abordagem complementar à estratégia de melhoramento e é provável também que seja necessária para garantir o sucesso da biofortificação genética, potencialmente melhorando tanto o impacto quanto a relação custo-eficiência dessas intervenções, especialmente em países de baixa renda

(CAKMAK, 2008; JOY et al., 2015; MELASH; MENGISTU; ABERRA, 2016; VELU et al., 2014; WHITE; BROADLEY, 2009).

No entanto, a introdução de alimentos biofortificados deve ser acompanhada por dispositivos técnicos, legais e estudos que comprovem sua necessidade, garantindo a segurança das populações de risco. Esses estudos devem abranger a identificação da prevalência da anemia, a avaliação periódica do estado nutricional e a adequação tecnológica para assegurar que o micronutriente esteja disponível e seja consumido pela população (VELLOZO; FISBERG, 2010).

As plantas cultivadas apresentam diferentes capacidades de acúmulo de nutrientes em partes comestíveis, variando em função do estádio de desenvolvimento, do método de aplicação do nutriente, características do solo e fontes de nutriente (SILVA; VITTI; TREVIZAM, 2007). Outro fator importante é a habilidade de remobilizar Zn das folhas para os grãos, dependendo do estado nutricional de Zn na planta que, por sua vez, é afetado pelas propriedades do solo. Além disso, uma parte do Zn aplicado nas folhas pode ser conduzido pelo sistema vascular e atingir a rizosfera onde a disponibilidade para a raiz da planta dependerá das propriedades do solo (JOY et al., 2015).

Mao et al. (2014) usando biofortificação agronômica para aumentar o teor de Zn, Se e I em partes comestíveis de cultivos verificaram diferentes conteúdos de Zn em soja (57-59 mg kg^{-1}), trigo (15 a 16), milho (16 a18), batata (13 a 15), canola (28 a 36) e repolho (20 a 61) quando submetidos a tratamentos envolvendo aplicação dos nutrientes via solo em conjunto e individualmente, evidenciando o potencial de acúmulo nos grãos de soja.

A forma de aplicação de Zn pode promover diferentes respostas no acúmulo do nutriente em diferentes espécies cultivadas. Joy et al. (2015) verificaram que aplicação de Znvia solo promoveu aumentos na concentração do nutriente em grãos de milho, arroz e trigo na ordem de 23, 7 e 19%, respectivamente, em comparação ao controle. Por sua vez, a aplicação via foliar promoveu aumentos de 30, 25 e 63% na concentração de Zn no grão, com maior efeito em plantas de trigo do que plantas de arroz na mesma condição (JOY et al., 2015).

Mao et al. (2014) verificaram que o sulfato de zinco aplicado via foliar é eficaz na biofortificação de trigo, contrariamente, o aumento da concentração de Zn em folhas de repolho e sementes de canola foi superior com o uso de Zn via solo.

Resultados diferentes foram encontrados por Jiang et al. (2013) onde aplicação de 200 mg kg^{-1} de Zn ao solo apresentou melhores resultados, promovendo o crescimento das folhas, grãos, melhorando as características de produção do trigo.

A aplicação foliar representa uma forma eficaz de melhorar a concentração de Zn em cereais. Seu uso em estádios mais avançados do desenvolvimento da planta em relação ao uso em estádios iniciais proporciona maiores incremento de Zn nos grãos, sugerindo que o Zn é facilmente translocado do floema para os grãos e que o uso da adubação foliar é uma eficiente estratégia para maximizar o acúmulo em partes comestíveis, como grãos (CAKMAK et al., 2010).

Embora eficiente para a biofortificação, a aplicação foliar com micronutrientes deve ser feita de forma segura para a planta, uma vez que pode proporcionar efeito fitotóxico. Martinez et al. (2009) verificaram que o aumento de doses de selênio para fins de biofortificação dos grãos de soja, promoveram redução na altura da planta e inserção do primeiro legume, causando visivelmente toxidez nas folhas das plantas, quando aplicado via foliar. Entretanto, a produtividade da soja não foi afetada pelas formas de aplicação e doses do elemento.

As adubações para fins de biofortificação podem ser combinadas com outras atividades empregadas no cultivo das plantas, como no uso, herbicidas, inseticidas e fungicidas. Dessa forma, pode-se otimizar os processos, reduzindo tempo e custo, melhorando a conscientização e adoção das estratégias de aplicação com Zn pelos agricultores (VELU et al., 2014).

Segundo Wanga et al. (2015), o sulfato de zinco pode ser misturado e pulverizado em conjunto com inseticida para controle de pulgão, sem causar qualquer efeito adverso, sendo útil e rentável para os produtores, contribuindo tanto para melhorar a produção e a concentração de Zn nos grãos quanto controlar efetivamente os insetos, reduzindo o custo e aumentando o rendimento líquido dos agricultores em 6,3% quando comparados a aplicação isolada de fertilizantes e inseticidas em diferentes momentos.

Algumas plantas contêm compostos nos grãos que reduzem a biodisponibilidade de Zn na dieta, como ácido fítico, lignina, cutina, suberina, tanino, ácido oxálico, metais pesados, entre outros (KING, 2002). Isso faz dos estudos sobre os fatores antinutricionais uma abordagem promissora e necessária, uma vez que reduzem a biodisponibilidade de nutrientes (GUPTA; RAM; KUMAR, 2016).

Welch et al. (2000) relataram aumento da biodisponibilidade de Fe e Zn para ratos alimentados com genótipos de feijão e arroz com maiores concentrações desses minerais.

A relação fitato/Zn é um indicador para a biodisponibilidade de Zn em dietas. A redução nessa proporção é um passo importante na melhoria da biodisponibilidade de Zn em humanos. A aplicação foliar de Zn pode ser efetiva na redução dessa relação sendo verificadas diminuição de 112 para 45 (fitato/Zn) em endosperma de trigo (CAKMAK et al., 2010).

Chomba et al. (2015) verificaram efeito favorável no consumo de milho biofortificado em crianças, apesar dos teores elevados na relação fitato/Zn, sugerindo que o consumo de milho biofortificado pode satisfazer os requisitos de Zn e proporcionar uma alternativa dietética eficaz, quando comparados ao consumo de milho não biofortificado em populações vulneráveis.

Processos envolvidos na industrialização também podem interferir nessa relação. Comparando diferentes frações de moagem em trigo, observou-se que a concentração de Zn e fitatos é significativamente maior em farelos, com grande diminuição na concentração e biodisponibilidade de Zn na extração de farinhas, tanto de grãos do tratamento controle quanto nos grãos de trigo biofortificados. A biodisponibilidade de Zn é maior na farinha oriunda de grãos biofortificado em comparação com a farinha controle e farinhas comercialmente disponíveis. Apenas a farinha de grãos integrais biofortificada pode assegurar melhor a biodisponibilidade de Zn para os grupos de populações humanas dependentes desse alimento (HUSSAIN et al., 2013).

Na produção de brotos de soja, o uso de solução com Zn em concentrações iguais ou menores 20 $\mu g\ mL^{-1}$ ($ZnSO_4$) mostrou-se viável, podendo ser uma boa fonte de Zn biodisponível produzido em curto período de tempo (ZOU et al., 2012).

Em geral, pode-se notar a variabididade existente entre cultivares de soja quanto as características agronômicas, teor de nutrientes e antinutrientes, sendo essas informações importantes para a seleção de material genético indicados para biofortificação. Um bom cultivar para esse fim deve apresentar altos teores de micronutrientes como o Zn e o Fe e baixo nivel de antinutrintes como o fitato (OLIVEIRA et al., 2016).

Entretanto, a maioria das pesquisas realizadas com Zn na cultura da soja estão relacionadas às melhores condições para o crescimento, desenvolvimento e produção de grãos. Assim, pesquisas abrangendo a temática da biofortificação, visando ao aumento dos teores encontrados no grão por meio da apropriada aplicação de Zn ou seleção de materiais genéticos com boa capacidade de acúmulo devem ser fomentadas.

Ressalta-se que embora a existência de excelentes resultados produzidos pelo uso da estratégia na diminuição das deficiências em micronutrientes, baseado em considerações de custo-eficiência, uma maior atenção deve ser dada para garantir que o Zn esteja disponível em cereais, a fim de gerar impacto na dieta das populações, garantindo também a segurança das populações-alvo (JOY et al., 2017).

Assim, é necessário o acompanhamento do uso dessas técnicas, por meio de dispositivos técnicos e legais e estudos que comprovem sua necessidade, de forma segura às populações de risco, por meio de avaliação periódicas do estado nutricional, assegurando que o micronutriente esteja disponível e seja consumido pela população-alvo pelo monitoramento constante nos programas de intervenção (VELLOZO; FISBERG, 2010).

2.4 Soja tipo-alimentação

A cultura da soja ocupa lugar de destaque na economia mundial, com produção de 312,67 milhões de toneladas e área plantada de 119,732 milhões de hectares no mundo. O Brasil é o segundo maior produtor mundial com uma produção de 113-114 milhões de toneladas na safra 2016/2017 e área plantada de 33.251 milhões de hectares. A China é o maior importador de soja do mundo (COMPANHIA NACIONAL DE ABASTECIMENTO - CONAB, 2016; UNITED STATES DEPARTMENT OF AGRICULTURE – USDA, 2016).

Segundo a Companhia Nacional de Abastecimento (CONAB, 2017), a projeção é de crescimento em 15,4% na produção de soja do Brasil. As produtividades alcançadas até o momento indicam um aumento em relação ao obtido na safra passada, podendo chegar a médias superiores a 3.500 kg ha^{-1} ou 4,8% superior.

A soja é cultivada praticamente em todo o território brasileiro, com destaque para os estados do Mato Grosso, Paraná, Rio Grande do Sul e Goiás como os maiores produtores. Apenas esses estados foram responsáveis por aproximadamente 74% de toda a produção nacional (CONAB, 2016).

A composição do grão de soja varia em função da cultivar e das condições de cultivo. Em geral, o grão da soja é constituído de 8% de cascas, 90% de cotilédones e 2% hipocótilos, sendo que os cotilédones contêm a maioria dos lipídios e proteínas, cerca de 60% na massa seca, seguidos de carboidratos (35%) e cinzas (5%) (LIU, 1999).

Além de boa fonte de proteínas e lipídeos, a soja possui fitoquímicos importantes em diferentes atividades metabólicas, como as isoflavonas. É rica em minerais como ferro, potássio, magnésio, zinco, cobre, fósforo, manganês e vitaminas do complexo B, sendo considerada um alimento funcional, por apresentar diversos benefícios à saúde, auxiliando na redução do risco de desenvolvimento de doenças crônicas e degenerativas (CIABOTTI et al., 2006; SILVA; CARRÃO-PANIZZI; PRUDÊNCIO, 2012).

O consumo da soja e seus derivados, fonte de isoflavonas, apresentam efeitos benéficos na prevenção do surgimento de doenças crônicas não transmissíveis como diabetes, hipertensão arterial e diversos tipos de câncer, além dos seus efeitos no controle dos sintomas da menopausa em mulheres. Assim, o consumo de soja deve ser incentivado, a fim de prevenir doenças futuras, garantindo uma melhor qualidade de vida (ZAKIR; FREITAS, 2015).

Estudos em cultura de células, modelos animais e alguns ensaios clínicos em humanos têm mostrado que as isoflavonas, encontradas em grandes quantidades na soja e em seus derivados, representam uma alternativa promissora na prevenção e/ou tratamento de cânceres relacionados ou não com hormônios, doenças cardiovasculares, osteoporose e alívio dos sintomas da menopausa (BEDANI; ROSSI, 2005).

A proteína de soja é a única proteína vegetal que contém todos os aminoácidos essenciais que suportam o crescimento e a manutenção de um organismo (AARON; MICHELFELDER, 2009). Segundo a lista de produtos com alegação de benefícios à saúde humana divulgada pela Agência Nacional de Vigilância Sanitária (ANVISA, 2005), o consumo diário de 25 g de soja ajuda na redução do colesterol, juntamente com dieta equilibrada e hábitos de vida saudáveis.

Dependendo das características do grão, a soja pode ser utilizada em diferentes produtos e finalidades, na indústria de alimentos, química e de energia (CANTELLI et al., 2017; SILVA; CARRÃO-PANIZZI; PRUDÊNCIO, 2009). Em países orientais, a soja é tradicionalmente utilizada em sua forma natural (nimame, edamame e brotos) ou na forma de produtos fermentados (natto, miso, tempeh, molhos, tofu e leite de soja fermentados). Nos países ocidentais, a soja é processada para produzir o óleo, proteína vegetal e biocombustíveis, sendo os resíduos da extração usados na preparação de rações para animais (CARVALHO et al., 2015; CASÉ et al., 2005; ROSA et al., 2009; ZAKIR; FREITAS, 2015).

A soja também é usada como ingrediente de diversos produtos industrializados, influenciando as características nutritivas, sensoriais, preparação, processamento ou estocagem dos alimentos, dada as suas características tecnológicas modificando a capacidade de hidratação, tamanho, forma e superfície das moléculas (GONÇALVES et al., 2014).

Observa-se, ainda, que mesmo com a grande produção agrícola brasileira, o consumo dos grãos e seus derivados pela população ainda é insatisfatório, isso se deve tanto à falta de informação com relação aos benefícios do seu consumo, quanto à falta de costume da população em consumir o grão como parte da alimentação usual (PENHA et al., 2007; ZAKIR; FREITAS, 2015).

Um fator limitante ao consumo de soja é o sabor desagradável, “off flavor”, causado pela oxidação e instabilidade de ácidos graxos, em decorrência da presença das enzimas lipoxigenases. Esse cenário estimulou o desenvolvimento de cultivares especiais, com baixa ação ou livres de lipoxigenases, soja “tipo alimentação”, garantindo propriedades químicas, físicas e sensoriais especiais, para diferentes usos e nichos de mercado, com o objetivo de melhorar a aceitação da soja na alimentação humana, diferentemente das cultivares “tipo grão” tradicionalmente utilizadas para a extração de óleo e nutrição animal (CIABOTTI et al., 2016; ESTEVES et al., 2010; SILVA; CARRAO-PANIZZI; PRUDÊNCIO, 2009).

Outras características importantes para a qualidade da soja tipo-alimentação é o tamanho dos grãos, alto teor de proteína, coloração clara do hilo, bons teores de açúcares, aminoácidos, ácidos orgânicos. Deve apresentar também teor reduzido do inibidor de tripsina Kunitz, permitindo a redução de tratamento térmico e dos custos de processamento, além de boa capacidade de absorção de água e menor tempo de cozimento (CHARLO et al., 2011; SONG; ANB; KIM, 2003).

No Brasil, dentre as principais cultivares de soja para alimentação humana, destacam-se: BRSMG 800A, BRSMG 790A, BRSMG 715A, BRS 257, BRS 21, BRS 267, BRS 216, BRS 258, BR 36, BRS 155, IAC PL-1 (BAVIA et al., 2012; CIABOTTI et al., 2006, 2016; CHARLO et al., 2011; CZAIKOSKI et al., 2012; SANTANA et al., 2012; SANTOS et al., 2013).

Uma boa opção de utilização da soja na alimentação, visando a qualidades funcionais, é por meio de grãos verdes, conhecidos como soja-hortaliça, soja-verde ou edame (quando submetida à cocção em água e sal) (MENDONÇA; CARRÃO-PANIZZI, 2003). A soja-hortaliça é uma fonte importante de proteína de baixo custo e outros nutrientes. É também uma adição valiosa a um sistema de cultivo, produzindo um rendimento biológico de até 40 toneladas ha^{-1} dentro de 75 dias (KEATINGE et al., 2011).

Embora se trate de variedades diferenciadas de soja, o sistema de cultivo da soja tipo-alimentação é o mesmo para a soja tipo-grão até o estádio reprodutivo R6 (legume com cavidades completamente cheias, de coloração verde) quando são colhidas. Após a colheita, é feito o destacamento e seleção dos legumes (com dois ou mais grãos), embalagem e comercialização. Outra opção é a colheita em estádio reprodutivo R8 (maturação plena). Assim, cultivares de tegumentos coloridos como a BRSMG 715A (tegumento preto) e BRSMG 800A (tegumento marrom) apresentam características que se assemelham ao feijão.

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1 - ZINC BIOFORTIFICATION STRATEGIES IN FOOD-TYPE SOYBEAN CULTIVARS

(VERSÃO PRELIMINAR)

Artigo elaborado segundo as normas da revista *Australian Journal of Crop Science*

Abstract

Reduce the risk of Zn deficiency by the diversification of the diet and agricultural interventions like agronomic biofortification remain a significant challenge for the future. This study was carried out in a greenhouse to investigate the effect of Zn fertilization strategies on soybean grains cultivars biofortification intended for human consumption. A completely randomized 5x3 factorial design, with four replications was used. Five Zn fertilization strategies were tested (control without application of Zn, via soil, via foliar in three growth stages, vegetative 4, vegetative 8 and reproductive 8) in three soybean cultivars (BRS 213, BRSMG 790A and BRS Favorita RR®). The Zn fertilization via foliar in R4 stage promotes greater Zn biofortification of soybeans grains in low availability of this nutrient in the soil. The cultivars showed different responses as Zn accumulation, the BRSMG 790A with highest increase of Zn in the grain. The use of fertilization with Zn benefits the protein content in grain as well as the plant height and number of grains per plant of soybean.

Keywords: *Glycine max* (L) Merril; green-soybean; micronutrient; plant nutrition.

Abbreviations: CARBO_ carbohydrate; DM100_hundred-grains dry matter; FM100_ hundred- grains fresh mass; FPM_fresh pod mass; IFP_insertion of the first pod; NGP_ number of grains per plant; NGP_number of grains per pod; NPP_number of pods per plant; PH_plant height; R4_reproductive growth stage; R6_reproductive growth stage RR_Roundup Ready; V4_vegetative growth stage 4; V8_vegetative growth stage 8.

Introduction

Nowadays, malnutrition still a problem, despite progress in reducing the prevalence of hunger in the world, more than 795 million people still unable to meet their daily needs for a healthy life. When available, sometimes the quality of the food does not have the nutritional requirements (vitamins and minerals), and therefore the population continues to suffer from nutritional deficiencies, such as zinc (Zn) (FAO, 2015).

It is estimated that with the continued expansion of the world population simultaneously with climate changes, urban sprawl and a decline in soil fertility, major agricultural impacts are expected. Thus, the challenge of providing nutritionally adequate diets to the population becomes increasingly difficult (Miller and Welch, 2013).

About half of the world's soils are deficient in Zn. Thus, the cultivation of cereals for human consumption in these soils promote low Zn concentration in produced foods (Das and Green, 2013). Incidence of micronutrients deficiencies in crops increased markedly in recent years because of intensive cropping, loss of topsoil layers by erosion, liming of acidic soils, decreased proportions of farmyard manure compared with chemical fertilizers, increased purity of chemical fertilizers, and use of marginal lands for crop production (Almeida Júnior et al., 2007; Fageria et al., 2012).

As a consequence, plants with Zn deficiency in general have low concentration of this nutrient in their tissues and it can promote a crop yield reduction. Products from these plants have a smaller contribution to the Zn content in the human diet. If the Zn concentration in staple foods are increased, great are the benefits on the consumption of Zn in the human diet (Alloway, 2008).

Among the possible solutions to correct the Zn deficiencies in human alimentation is the crop biofortification with important elements for human health and it has been advocated as a public health strategy to address mineral deficiencies in humans (Cakmak, 2008; White and Broadley, 2009; Zimmermann, 2013; Joy et al., 2015).

The biofortification can be achieved by two different techniques, an agronomic (fertilization) or genetic biofortification (breeding) (Cakmak, 2008). Plant Agronomic intervention by Zn fertilizers application, either through leaf routes or by soil, can contribute to the increase of Zn content in food crops (Joy et al., 2015). Similarly, plant breeding through the development of new cultivars with the ability to uptake and accumulate more Zn as well as translocate it to edible parts may potentially be an efficient way to increasing the bioavailability of the nutrient (Raboy, 2009).

In order to maximize the effect of Zn in the production, the way of application is fundamental to assure greater plant absorption of the nutrient by the plant (Correia et al., 2008). Studying the availability of Zn to soybean growth Han et al. (2011) verified that Zn application via seed coating and foliar sprays resulted in greater efficiencies when compared to soil (side dressing or incorporated). However, few researches are found when the goal is the biofortification of grains and not the growth and production of soybean.

Nowadays, soybean [*Glycine max* (L.) Merrill] is the most important crop source of hight quality protein and other nutrients, mainly in diets with animal products restriction. It is a nutritionally rich food, serving to organism nutrition, helping to maintain the health of the population (Penha et al., 2007; Keatinge et al., 2011).

One factor that limits soybean consumption is the off flavor, which occurs by fatty acids instability and oxidation. Thus, soybean cultivars that are free or with reduced levels of the lipoxygenase enzymes, responsible for this characteristic, are being developed to guarantee a better grains flavor, promoting the increase of consumption (Esteves et al., 2010). An interesting option for the use of soybeans in human foods is by green grains, known as green-soybean, soybean-vegetable or edamame (vegetables cooked in water and salt) (Mendonça and Carrão-Panizzi, 2003). Although, it is a differentiated soybean cultivar, the green-soybean cultivation system is the same as the common soybeans up to the reproductive stage R6, with the pod completely filled and the green color, when harvesting is performed. After the harvest is made the selection of vegetables with two or more grains.

Thus, reduce the Zn nutritional deficiency risks through dietary diversification and agricultural interventions such as the use of biofortification represent a significant challenge for the future (Kumssa et al., 2015).

Therefore, the aim was to evaluate the effect of different ways of Zn fertilization on biofortification of soybean cultivars recommended for human consumption.

Materials and Methods

Plant material

The experiment was carried out from November 2013 to March 2014, in greenhouse at Crop Science Department of the Federal University of Lavras, Minas Gerais, Brazil, located at 21°14’S latitude, 45°00’W longitude and altitude of 918 m. Pots with 5 dm^{-3} were used, filled with samples of the layer 0-20 cm of a Red Latosol, medium texture, whose chemical analysis result was: pH in water: 5.4, K: 20 mg dm^{-3}, P (Mehlich-1): 0.84 mg dm^{-3}, S: 1.56 mg dm^{-3}, Ca: 0.32 $cmol_c$ dm^{-3}, Mg: 0.10 $cmol_c$ dm^{-3}, Al: 0.10 $cmol_c$ dm^{-3}, potencial acity (H+Al): 2.90 $cmol_c$ dm^{-3}, organic matter (O.M): 2.36 dag kg^{-1}, Fe: 22.53 mg dm^{-3}, Mn: 3.88 mg dm^{-3}, Cu: 1.88 mg dm^{-3} e B: 0.13 mg dm^{-3}. The total content of Zn found in this soil was 0.33 mg dm^{-3}, classified in the low content range for soybean crop according to the limits established by Galrão (2002).

Experimental design and Zn strategies

It was used a completely randomized design in a 5x3 factorial arrengement with four replicates. Five Zn fertilization forms: via soil, via foliar in V4 (four fully developed trifoliolate leaves), foliar V8 (eight fully developed trifoliolate leaves), foliar in reproductive R4 (full pod) and control. Three soybean cultivars was used, BRS 213, BRSMG 790A and BRS Favorita RR®, with four replicates. Each experimental unit consisted of two plants per pot. The cultivars BRS 213 and BRSMG 790A are recommended for human consumption (food-type) and BRS Favorita RR® recommended for industry.

Based on the soil chemical analysis, the corrections of Ca and Mg with dolomitic limestone were made according to the base saturation method, considering the level of 70% of ideal saturation. After 30 days, the soil fertilization was performed according to Malavolta (1980) using 200 mg P dm^{-3} (single superphosphate), 150 mg K dm^{-3} divided into two applications (potassium chloride) and 50 mg S dm^{-3} (single superphosphate). The fertilization with micronutrients, except Zn, was carried out near the planting time, with 7 mg of Mn being applied; 3 mg Cu; 0.5 mg B; And 0.2 mg of Mo per dm^3 of soil, supplied as manganese chloride, copper sulfate, boric acid and ammonium molybdate.

The level of 3 mg Zn per dm^{-3} of soil was used as source zinc sulphate (23% of Zn) following the recommendation of Sfredo (2008). In soil treatment, Zn was applied at the time of planting. In the treatments with foliar fertilization was used 1.82 mg L^{-1}. Before the application, the required amount of solution to complete wetting was simulated in function of the different plant sizes for each stage of development, avoiding drainage to the soil. The applications were made in individual pots using a manual pre-pressure sprayer.

Conduction of experiment

Were used six seeds per pot. After the first trifolium appeared (V1) the thinning was carried out maintaining two plants per pot. During cultivation, irrigation was used by controlled microaspersion, maintaining the soil with the field capacity in 60% humidity.

When necessary, pest and disease control was applied with the application of the insecticide teflubenzurom at a rate of 0.05 L of pc ha^{-1}, application of fungicide, azoxystrobin + ciproconazole at a rate of 0.3 L of pc ha^{-1} + An additional 0.5% of the Nimbus® adjuvant. Weed control was performed manually.

Traits measured

Data collection was performed when the plants were in reproductive stage R6 (pod containing green seed that fills the pod cavity at one of the four uppermost nodes on the main stem with a fully developed leaf). The following agronomic and productive parameters were determined: plant height an insertion of 1st pod (cm); fresh mass of pods per plant (g); number of pods per plant; number of grains per plant; number of grains per pod; hundred-grains fresh mass (beans with a mean moisture content of 53%); hundred-grains dry mass (average moisture of 13%). For the determination of the Zn content and grain composition, samples were previously oven dried with forced air circulation at 65°C until reached a constant mass and were later ground in a knife mill. The methodology described by Malavolta et al. (1997) was used to determine the total Zn contents (mg kg^{-1}). The centesimal composition of the grains through the quantification of moisture, ashes, proteins, lipids, crude fiber and carbohydrates were determined according to AOAC (2006).

Statistical analysis

The data were submitted to variance analysis and when a significant difference was verified by the F test, the means were grouped by the Scott-Knott test ($p < 0.05$) using SISVAR software (FERREIRA, 2011).

Results and Discussion

Soybean traits results

The interaction between Zn fertilization strategies and soybean cultivars was significant for the traits zinc, lipid and protein in the grain (Table 1). The agronomic parameters insertion of the first pod, number of pods per plant, number of grains per pod, fresh pod mass and hundred-grains and dry matter of hundred-grains, as well as the ash content of were influenced by cultivars. The parameters plant height, number of grains and carbohydrate content were influenced both by the forms of fertilization with Zn and by the cultivars. There was no statistical difference in the fiber content, presenting a general average of 6.76% of the grain composition as function of the used treatments.

Soybean grain biofortification and composition

It was observed that all treatments with Zn application altered the content of this nutrient in the grain in all cultivars studied when compared to the control. The content of Zn in the grain varied from 23.93 to 49.17 mg kg^{-1} (Table 1). The highest levels were found when the application of Zn was performed by foliar at the R4 growth stage. This stage is characterized by the fast growth of the pod and the beginning of grain development, preceding a period of fast and constant accumulation of dry matter, which increases the nutritional demand of the plant. Carvalho et al. (2015) evaluating the accumulation of Mn in soybean grains, observed a greater accumulation when the nutrient was applied at the reproductive R3 stage compared to the application at the R1 stage, maybe related to the period of accumulation of grain reserves, because the application in R3, beginning of the pod formation, was carried out closer to the filling of the grains in relation to R1, beginning of flowering.

The cultivar BRSMG 790A presented the highest accumulation of Zn in the grain with the foliar application in the R4 stage reaching 49.17 mg kg^{-1} (Table 1). The use of Zn foliar fertilization in R4 provided increases on the Zn content of the grain in the order of 56% in the cultivar BRS 213, 105% in the BRSMG 790A and 36% in the BRS Favorita RR® when compared to the contents found in the control. It suggest that the studied soybean cultivars have differences in Zn uptake capacity and translocation for the grain, but they have responded positively to the application strategies used for the biofortification of the grain. BRSMG 790A, intended for human consumption, presented the highest absorption and translocation capacity of Zn for the grain and, therefore, the highest nutrient content, followed by BRS 213.

Oliveira et al. (2016) evaluating the genotypic variation of the Zn content in grains of 24 soybean cultivars, aiming identify cultivars with potential for biofortification, found a mean content of 40 mg kg$^{-1.}$

The greater availability of Zn in the grain derived from foliar applications when compared to soil fertilization, demonstrates that this form of biofortification is an attractive and useful strategy to solve health problems related to nutrient deficiency (Cakmak, 2008). Joy et al. (2015) suggested that the cost-benefit of Zn foliar application in wheat seems to be equivalent to the fortification of basic flours, common stage in the industrialization process.

In wheat research was observed foliar applied Zn was more effective than soil applied Zn for biofortification with the maximum grain Zn of 30 mg kg^{-1} (Mao et al., 2014).

Table 1. Mean values for Zn concentration, lipid, protein and fiber content of soybean cultivars intended for human consumption using forms of Zn fertilization, agricultural year 2013/14, Lavras, MG, Brazil.

Cultivar	Control	Soil	Foliar V4	Foliar V8	Foliar R4
			Zinc (mg kg^{-1})		
BRS 213	27.29 b D	36.31 b B	30.11 c C	36.13 a B	42.56 b A
BRSMG 790A	23.93 c D	35.13 b B	33.15 b C	33.75 b B	49.17 a A
BRS Favorita RR®	31.86 a D	39.74 a B	35.05 a C	35.71 a C	43.44 b A
CV(%)			3.97		
			Lipids (%)		
BRS 213	13.61 b A	15.87 b A	14.30 b A	15.48 a A	15.33 a A
BRSMG 790A	18.30 a A	18.12 a A	15.70 b B	13.05 b C	16.40 a B
BRS Favorita RR®	16.87 a A	16.87 b A	17.20 a A	17.06 a A	17.40 a A
CV(%)			5.51		
			Protein (%)		
BRS 213	38.78 a B	39.16 a B	39.71 a B	39.22 a B	46.11 a A
BRSMG 790A	35.14 a B	35.18 b B	37.77 a A	40.97 a A	38.55 b A
BRS Favorita RR®	37.57 a B	41.17 a A	40.50 a A	40.67 a A	42.32 b A
CV(%)			3.65		

Means followed by the same lower case letter in the column and upper case in the line are from the same group according to Scott-Knott test ($p \leq 0.05$).

Regarding the lipid content, only the cultivar BRSMG 790A showed a significant difference with Zn application. The highest levels were found when Zn was not used (18.30%) and when the nutrient was applied by soil (18.12%) (Table 1). For the cultivars BRS 213 and BRS Favorita RR® the lipids content did not present a significant difference as a function of the Zn application, remaining on average with 14.89 and 17.08% of lipids in the grain, respectively.

The protein content increased by the foliar application in R4 when compared to the control. The cultivar BRS 213 showed the highest protein content with 46.11% along with BRS FAVORITA RR® (42.32%), differing from BRSMG 790A, which presented 38.55% when Zn was applied in R4 (Table 1).

Among the functions of Zn in plants is the synthesis of proteins, which may have contributed to the occurrence of positive results in grain protein content as a function of Zn application. Ash and carbohydrate contents presented statistical difference only among cultivars studied (Table 2). The highest ash content was verified in cultivar BRS 213 (5.98%) differing from cultivars BRSMG 790A (5.76%) and BRS Favorita RR® (5.56%). The BRS 213 and BRSMG 790A presented the highest carbohydrate content with 25.89 and 26.75%, respectively, BRS Favorita RR® presented 23.87%, independent of Zn fertilization.

It was verified that the carbohydrate contents were similar in the treatments control (27.75%), by soil (25.48%), by foliar V4 (26.29%) and foliar V8 (25.66%), except when Zn was applied via foliar in R4, presenting the lower content of 22.34%) (Table 3).

Vieira et al. (1999) studying the centesimal composition of six soybean cultivars for human consumption found that all cultivars studied had a typical centesimal composition of commercial soybean (type grain), with a mean dry base content of 39.52% Protein, 23.04% oil, 5.41% ash, 5.75% fiber and 32.01% total carbohydrates. Ciabotti et al. (2006) did not observe a difference in the centesimal composition of BRS 133, common soybean cultivars, and BRS 213 lacking lipoxygenases (food use), presenting a mean content of 32.7% of proteins, 15.7% of lipids and 30.0% of carbohydrates in the conventional cultivar and 33.2%, 15.3% and 31.1%, respectively in cultivar with lipoxygenases.

The availability of nutrients to the plant may influence the chemical composition of the grains. Thus, the adequate supply of nutrients favors the development of the plants, conditioning them to produce the necessary metabolites in the development of grains or seeds (Veiga et al., 2010).

Strategies such as the use of fertilizers provide an immediate and effective option to increase Zn concentrations in grains and yields, particularly in soils with Zn deficiency. When combined with other managements such as agrochemicals or even combined with the use of herbicides, fungicides or insecticides, Zn foliar application may be feasible, reducing time and costs (Velu et al., 2013).

Growth traits and yield

Comparing the height plant results among cultivars, BRS Favorita RR$^{®}$ shows the higher height (55.56 cm), differing from BRSMG 790A (45.72) and BRS 213 (41.93) (Table 2). In relation to fertilization with Zn, all treatments that received the nutrient showed similar height differing from the average found in the control treatment.

Table 2. Mean values of plant height (PH), insertion of the first pod (IFP), number of pods per plant (NPP); number of grains per pod (NGP), fresh pod mass (FPM), hundred- grains fresh mass (FM100), hundred-grains dry matter (DM100), carbohydrate content in the grain (CARBO) and ash content in the (ASH) of soybean cultivars for human consumption as a function of Zn fertilization, agricultural year 2103/14, Lavras, MG, Brazil.

	BRS 213	BRSMG 790A	BRS Favorita RR$^{®}$	CV (%)
PH (cm)	41.93 c	45.72 b	55.56 a	10.71
AFP(cm)	8.51 c	14.00 b	15.45 a	21.79
NPP (un)	26.82 a	26.72 a	22.00 b	26.92
NGP (un)	2.29 b	2.09 b	2.51 a	23.10
FPM (g)	27.27 a	22.60 b	29.10 a	32.97
FM100 (g)	29.34 b	28.28 b	31.09 a	11.38
DM100 (g)	13.22 b	14.34 a	13.17 b	16.34
CARBO (%)	25.89 a	26.75 a	23.87 b	7.19
ASH (%)	5.98 a	5.76 b	5.56 b	3.75

Means followed by the same lower case letter in the line are from the same group according to Scott-Knott test ($p \leq 0.05$).

The same behavior was observed at the insertion of the first pod (Table 3). The cultivar BRS Favorita RR$^{®}$ had a higher insertion height with 15.45 cm, followed by BRSMG 790A with 14.0 cm and BRS 213 with 8.51 (Table 2). The lowest number of pods per plant was found in BRS Favorita RR$^{®}$ (22.00). The cultivars BRS 213 and BRSMG 790A presented similar averages with 26.82 and 26.72 pods per plant.

For the number of grains per plant there was no statistical difference between the cultivars of soybean, however, in relation to the fertilization with Zn was observed that the treatments that contained this element, applied by soil or foliar, presented similar and superior averages comparing with found in the control treatment (Table 3). The variation found was from 48.62 (control) to 59.66 (foliar in V4) grains per plant. Evaluating some soybean genotypes Santos et al. (2013) found similar values, with averages of 40 to 67 grains per plant.

Table 3. Mean values of plant height (PH), number of grains per plant (NGP) and carbohydrate content (CARBO) of soybean cultivars for human consumption as a function of Zn fertilization, agricultural year 2103/14, Lavras, MG, Brazil.

	PH (cm)	NGP (un)	CARBO (%)
Control	44.00 b	48.62 b	27.75 a
Soil	48.90 a	58.66 a	25.48 a
Foliar V4	48.00 a	59.66 a	26.29 a
Foliar V8	49.70 a	55.08 a	25.66 a
Foliar R4	48.00 a	56.41 a	22.34 b
CV (%)	10.71	21.43	7.19

Means followed by the same lower case letter in the column are from the same group according to Scott-Knott test ($p \leq 0.05$).

The cultivar BRS Favorita RR® presented higher number of grains per pod with 2.51 differing statistically from BRS 213 (2.29) and BRSMG 790A (2.09). The highest fresh mass of hundred-grains was found in BRS Favorita RR® (31.09 g) when compared to the other cultivars. The cultivar BRS 213 and BRSMG 790A had equal results with 29.34 and 28.28 g respectively. Charlo et al. (2011) proposes that the greater mass of the grains characterizes a desirable factor in genotypes of green-soybean, being a characteristic that indicates materials with greater productions of green grains, being reported in the literature values between 28 and 56 g. However, Smiderle et al. (2011) studying performance of 8 cultivars found averages of up to 95 g for fresh mass of hundred-grains. The BRSMG 790A presented the highest average for the dry mass of 100 grains (14.34 g).

The results related to the Zn function in the plant were expected, since it is necessary for the production of tryptophan, amino acid precursor of indole acetic acid, vegetal hormone of growth, being also involved in the metabolism of nitrogen, being necessary for the maintenance of the membrane integrity (Malavolta, 2006). It was observed that the use of Zn fertilization, either by soil or by foliar route, brought benefits to the height and number of grains per plant when compared to the results found in the control treatment, evidencing the importance of fertilization strategies with Zn in soils with low nutrient content (Table 3).

Inocêncio et al. (2012) studing strategies with Zn increased soybean yield, even in soil with a micronutrient content above the critical level, showing that the productivity obtained has a significant impact on the dynamics and in Zn stocks in the soil-plant system. Thus, the restitution of the exported nutrient must be constantly considered in fertilization programs.

The productive capacity of genotypes and their influence on the concentration of nutrients in edible parts are extremely important when the objective is biofortification. Oliveira et al. (2016) found variability in the yield, height of plant, height of insertion of the first pod, as well as iron, zinc and phosphorus content in the grains among 24 soybean cultivars studied for selection of genotypes for biofortification.

Moreover, the use of foliar fertilization with Zn improve some agronomic characteristics of soybean plants and also increase the Zn content in the grain guaranteeing biofortification. Correcting Zn deficiency in plants, yields are improved, further contributing to nutritional improvement and quality of food crops, and consequently to human nutrition (Das and Green, 2013).

The use of fertilizers to increase the zinc content in cereals (agronomic biofortification) requires modifications in terms of quantity and frequency application of zinc sources, however, it is important that the nutrient concentrations in the soil should be monitored in order to avoid excessive accumulation, which may affect the soil biology even before the observation of phytotoxic effect on the crops. In this way, foliar fertilization with micronutrients, such as Zn, presents a lower risk of exceeding the maximum safe and permissible concentrations (Alloway, 2008).

Conclusion

According to the conditions that this study was carried out and the results, Zn fertilization via foliar in R4 stage promotes the greater soybean grain biofortification under low availability of this nutrient in the soil. The cultivars present distinct responses regarding the accumulation of Zn, with emphasis to BRSMG 790A cultivar with the highest Zn increase in grains. Moreover, the use of Zn fertilization provides increases in protein content in grains, as well as the plant height and number of grains per plant.

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ARTIGO 2 - BIOFORTIFICATION WITH ZINC RATES IN FOOD-TYPE SOYBEAN CULTIVARS

(VERSÃO PRELIMINAR)

Artigo elaborado segundo normas da revista Sociedade de Ciências Agrárias de Portugal.

ABSTRACT

The objective of this research was to verify the effect of zinc rates applied via foliar in food-type soybean cultivars aiming the biofortification of the grains. The experiment was set up according to a completely randomized design with four replicates, in a 5x5 factorial scheme: five soybean cultivars, BRS 010 (black tegument), BRS 213 (yellow), BRSMG 790A (yellow), BRSMG 800A (brown) and Favorita RR® (yellow) combined with five zinc foliar rates applied at the reprodutive 4 stage (0, 0.91, 1.82, 2.73 and 6.37 mg L^{-1}. The evalueted parameters was plant height, height of the first pod, number of pods per plant, number of grains per plant, pod mass per plant, mass of grains per plant, mass of hundred-grains, mineral composition of the grains, lipid and protein content. Foliar fertilization with zinc rates increased plant height, grain mass and protein content in soybean grains. The use of zinc rates provide significant increase in zinc content in the grain independent of the studied cultivar, presenting increasing responses in the zinc content as a function of the zinc rate applied.

Keywords: fertilization, foliar application, mineral composition, micronutrients.

INTRODUCTION

Micronutrients deficiencies are common in human. Approximately one third of the world's population (2 billion people) suffers from vitamins deficiencies, particularly A and C, and minerals such as zinc (Zn), iron (Fe) and iodine (I) (FAO, 2015).

Zn deficiency in edible part of crops, grains for example, in part is due their unavailability by factors such as high soil pH, concentration of other ions, moisture, fertilizers addition and other conditions prevailing in most areas (Alloway, 2009). Another factor that contributes to Zn unavailability is plant inability to moving Zn absorbed from the roots to the grains (Sharma *et al.*, 2013)

Agronomic biofortification through the use of agricultural tools such as the application of fertilizers containing Zn, promoting the enrichment of food crops in the short term, and should be a public health strategy in countries with a high incidence of nutrient deficiency (Joy *et al.*, 2015).

Another alternative is based on plant breeding, genetic biofortification, providing a sustainable and cost-effective solution, by traditional methods such as genotype selection with better nutritional value or through genetic engineering. Therefore, both strategies can increase the concentration of minerals in edible portions and improve crop yields in low fertility soils (White and Broadley, 2009). In a long term, agronomic biofortification is a complementary approach to breeding strategies and it is probably necessary for the success of breeding efforts (Cakmak, 2008).

Soybean [*Glycine max* (L.) Merrill] is an important source of protein, providing good amounts of nutrients (Keatinge *et al.*, 2011). When compared to others important crops in the world, it stands out for the high nutritional content, presenting high quality protein and containing all the amino acids necessary for the growth and maintenance of organisms (Gandhi, 2009). Currently, soybean breeding programs have been searching for productive materials, with good agronomic characteristics and capable of expressing their nutritional quality, food-type cultivars, aiming at human alimentation.

Agronomic biofortification studies aimed at Zn, Se and I increasing contents through soil fertilization showed the ability of soybean to accumulate Zn in the grains, presenting levels between 57 and 59 mg kg^{-1}, higher than the content found in wheat (15 to 16 mg kg^{-1}), potato (13 to 15 mg kg^{-1}), canola (28 to 36 mg kg^{-1}) and cabbage (20 to 61 mg kg^{-1}) under the same experimental conditions (Mao *et al.*, 2014). This highlights the importance of this crop as a source of nutrients to diets and use in biofortification programs.

Foliar application of zinc represents an effective way to improve Zn concentration in cereals. The use of Zn foliar fertilization 0.5% (w/v) in more advanced growing stages of the crop in relation to the use in the early stages provides a larger increase of Zn in edible parts, as in grains, evidencing the use of this technique as an important strategy to maximize the Zn accumulation (Cakmak *et al.*, 2010).

However, a greater understanding of control over nutrient supply in the development of improved cultivar grains that may respond favorably to maintenance of grain yield and quality in adverse environments is necessary, thus avoiding excessive nutrient use or even the toxic effect the plants. Thus, the objective of this research was to verify the effect of Zn rates applied via foliar in food-type soybean cultivars aiming the grain biofortification.

MATERIAL AND METHODS

The experiment was carried out in a greenhouse, from November 2014 to March 2015 at the Crop Science Department of the Federal University of Lavras, Minas Gerais, Brazil, located at 21°14’S latitude, 45°00’W longitude and altitude of 918 m. Plastic pots with capacity of 7 dm^3, were filled with 0-20 cm samples of the medium-textured Red Latosol, whose characterization of the chemical analysis result was: pH in water: 5.6, K: 14 mg dm^{-3}, P

(Mehlich-1): 0.56 mg dm^{-3}, S: 9,48 mg dm^{-3}, Ca: 0.1 cmolc dm^{-3}, Mg: 0.10 cmolc dm^{-3}, Al: 0 cmolc dm^{-3}, potencial acity (H+Al): 2.08 cmolc dm^{-3}, organic matter (O.M): 1.41 dag kg^{-1}, Fe: 9.17 $mgdm^{-3}$, Mn: 1.11 mg dm^{-3}, Cu: 0.76 mg dm^{-3}, B: 0.15 mg dm^{-3} and Zn: 0.26 mg dm^{-3}. The total content of Zn found in this soil is classified in the low content range for the soybean crop.

Based on the chemical analysis, the soil was corrected to increase the saturation by 70% using dolomitic limestone (PRNT 80.41%). After a 30 days incubation period, keeping the humidity at 60%, the fertilization was performed using 200 mg of P dm^{-3} and 50 mg of S dm^{-3} ((H_2PO_4) 2.H_2O + $CaSO_4$.2H_2O), 150 mg of K dm^{-3} divided into two applications (KCl). Fertilization with micronutrients was also performed using 7 mg of Mn ($MnCl_2$.4H_2O); 3 mg Cu ($CuSO_4$.5H_2O); 0.5 mg B (H_3BO_3); and 0.2 mg Mo dm^{-3} soil ($NH_4$6Mo7$O_2$4.4H_2O).

The experiment was set up according to a completely randomized design with four replicates, in a 5x5 factorial scheme: five soybean cultivars, BRS 010 (black tegument), BRS 213 (yellow), BRSMG 790A (yellow), BRSMG 800A (brown) and Favorita RR® (yellow) combined with five zinc foliar rates applied at the R4 stage (0, 0.91, 1.82, 2.73 and 6.37 mg L^{-1}). The soybean cultivars studied are indicated for human consumption (food-type), as they have different characteristics regarding composition and taste. Only BRS Favorita RR® cultivar, for industrial use, is not recommended for this purpose.

At the planting, five soybean seeds were used per pot. After the first trifolium appeared, the thinning was carried out maintainig two plants. Was used irrigation by controlled microaspersion, maintaining the field capacity of 60% humidity.

The foliar Zn fertilization was made using $ZnSO_4$.7H_2O. Before the application was simulated the necessary amount of solution for complete plant wetting, avoiding drainage to the soil. The applications were made in individual pots using a manual pre-pressure sprayer.

The control of pests and diseases was carried out with application of the insecticide teflubenzurom at the rate of 0.05 L of pc ha^{-1}, applied at 35 days after emergence (DAE) and with fungicide use, azoxystrobin + ciproconazole at a rate of 0.3 L of pc ha^{-1} plus an additional 0.5% of the Nimbus® adjuvant applied at 50 DAE. Weed control was performed manually.

Data collection was started when the plants reached the reproductive development stage R6 (full grain) with the evaluation of plant height and height of the first pod. In order to study the other parameters, the plants were carried to the reproductive stage R8 (full maturmaturity), and yield components were measured: number of pods per plant, number of grains per plant, pod mass per plant, mass of grains per plant and mass of hundred-grains. Grains mineral composition, lipid and protein content, grain samples were previously oven dried with forced air circulation at 65° C until reached a constant mass and were later milled in a knife mill. The mineral composition was performed with the use of Inductively Coupled Plasma Mass Spectrometry (ICP-MS, model iCAP 600, Thermo Sientific, Cambridge, United Kingdom) using 100 mg finely ground grains, pre-digested in 4 ml Of $HNO_3/HClO_4$ (60/40%, v / v) according to protocol described by Lyi *et al*. (2005). The lipid and protein content of grains were determined according to the methods of AOAC (2006).

The datas were submitted to variance analysis and regression. The choice of the model for each parameter was performed according to the significance of the coefficients and the R^2 values. In the isolated significance of the cultivar factor, the means were compared by the Scott-Knott test (P <0.005) using SISVAR software.

RESULTS AND DISCUSSION

Through variance analysis there was significant effect of cultivar on plant height, insertion height of first pod, yield components and mineral composition. Differences between zinc fertilization rates were verified in the traits plant height, grain mass, lipid content and protein content being analyzed the simple effect. The interaction between Zn fertilization rates and soybean cultivars was significant only for the trait zinc content in the grain.

Plants of the cultivars BRS Favorita RR® and BRSMG 800A presented higher plant height and height of the first pod insertion with approximately 62 cm and insertion above 12 cm (Table 1). The cultivars BRS 010 and BRS 213 had lower values for these variables. The ideal plant height is between 60 and 110 cm and insertion equal or greater than 10 cm, which in commercial crops may facilitate mechanical harvesting and avoid bedding. In this way, the cultivars BRS 010 and BRS 213 presented inferior results to the desirable range, under this experiment conditions.

On parameter number of pods per plant (Table 1) the cultivar BRS Favorita RR® presented about 38 pods per plant differing statistically from the other cultivars. The same result was verified for number of grains per plant (83.48 grains). The cultivar BRS 213 showed lower number of pods and grains with 27.69 and 55.59, respectively.

For the mass of pods the best average was presented by cultivar BRSMG 800A (20.25 g) differing from cultivars BRS 010 and BRS 213, which had the lowest averages (14.36 and 13.80 g) (Table 1). However, BRS Favorita RR®, BRSMG 800A and BRSMG 790A did not show any differences between them, with averages of 12.21, 12.19 and 11.19 g, respectively. There was a statistical difference between this group when compared to cultivars BRS 010 (9.86 g) and BRS 213 (9.35 g).

The parameter mass of hundred-grains is an important trait in relation to the food-type cultivars (Table 1). In this sense, the highest average was obtained in cultivar BRSMG 800A with 18.96 g, followed by cultivars BRS 213 and BRSMG 790A with 17.47 and 17.19 g, respectively. The lowest mean was found in cultivar BRS 010 with 13.71 g.

Table 1- Mean of plant height (PLH), height of the first pod insertion (HFP), number of pods per plant (NPPL), number of grains per plant (NGPL), mass of pods per plant (PMPL), mass of grains (GMPL) and mass of hundred-grains (M100G) of food-type soybean cultivars. Lavras, MG, Brazil.

	PLH	HFP	NPPL	NGPL	PMPL	GMPL	M100G
	--------cm-------		------un------		----------g----------		
BRS 010	49.55 c[1]	9.85 c	33.91 b	72.61 b	14.36 c	9.86 b	13.71 d
BRS 213	50.39 c	9.56 c	27.69 c	55.59 c	13.80 c	9.35 b	17.47 b
BRSMG 790A	54.72 b	12.01 b	33.89 b	68.83 b	16.99 b	11.29 a	17.19 b
BRSMG 800A	62.22 a	14.06 a	35.85 b	69.64 b	20.25 a	12.19 a	18.96 a
BRS Favorita RR®	62.02 a	12.37 b	38.14 a	83.48 a	17.89 b	12.21 a	15.76 c
CV(%)	5.51	15.58	13.33	14.89	13.7	12.95	7.61

[1] Means followed by the same lower case letter in the column are from the same group according to Scott-Knott test ($p \leq 0.05$).

The height of the plants was also influenced by the increase of Zn rates applied via foliar (Figure 1A). The use of the rate 2.73 mg L^{-1} provided greater increases in plant height when compared to 0 mg L^{-1}. The increase in Zn rates also had the same effect on the grain mass per plant (Figure 1B). The largest increment was found using the rate 2.73 mg L^{-1}. Although foliar Zn application has been carried out at the R4 stage, a reproductive development stage, the stabilization of growth in stature of soybean plants is generally achieved at the R6 (fully filled vegetable) stage. It is during this stage that the plant reaches the maximum mass accumulation of pods with high accumulation of dry mass and nutrients, both in the plant and in the grains (Bonato, 2000).

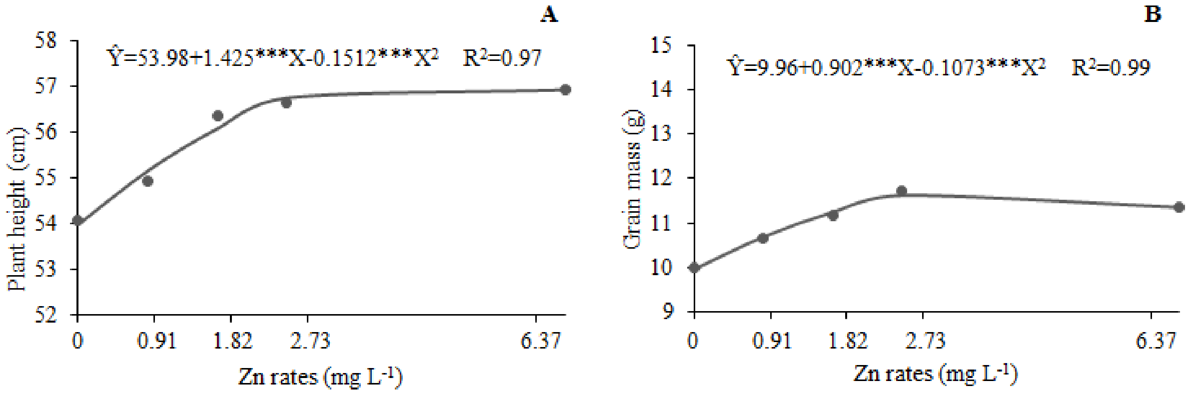

Figure 1- Plant height (A) and grain mass per plant (B) as a function of Zn fertilization rates.

Regarding the grain composition, the highest lipid content was presented by the BRS 010 cultivar with 20.10% in dry mass of grains, differing from the other cultivars (Figure 2A). By studying the isolated effect of Zn rates under the lipid content in the grain, a decreasing linear effect was observed as a function of the Zn rate increase. As for the protein content, the response was quadratic (Figure 2B). The highest Zn content was approximately 45% with the use of 2.73 mg L^{-1} of Zn.

In studies carried out with soybean grains recommended for human consumption, were observed values between 16 to 22% of lipids and 23 to 43% of protein in the grains (Gonçalves *et al*., 2014; Ciabotti *et al*., 2016).

Zn is an essential element and is involved in several physiological processes of plant growth and metabolism, including protein synthesis and lipid metabolism. The correlation between protein and oil content is considered negative, because as protein content increases, the oil content is reduced and vice versa (Moraes *et al*., 2006).

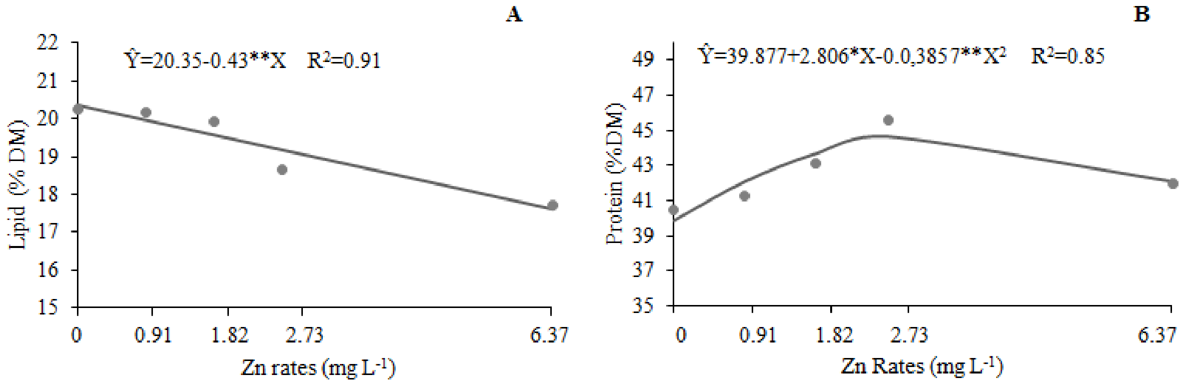

Figure 2- Lipid (A) and protein (B) concentration in soybean grains as a function of Zn rates fertilization via foliar.

The use of Zn rates provide significant increase in zinc content in the grain independent of the studied cultivar, presenting increasing responses in the Zn content as a function of the Zn rate applied (Figure 3). The highest zinc content in the grain was obtained by the cultivar BRSMG 790A combined with the foliar application of 6.37 mg L^{-1} presenting about 64 mg g^{-1}, representing an increase in order of 80% more than the content found in the control treatment (0 mg L^{-1}).

In some crops the foliar application of zinc presents better results than in others. According to Zhao *et al.* (2011) Zn foliar application in wheat plants may have little effect on increasing the Zn concentration in grain if the plant can absorb sufficient Zn through the soil solution. Zn in rice grain can be effectively increased by foliar Zn application (0.5% zinc sulfate) in particular when was sprayed after flowering promoting agronomic and nutritional benefits (Boonchuay *et al.*, 2013)

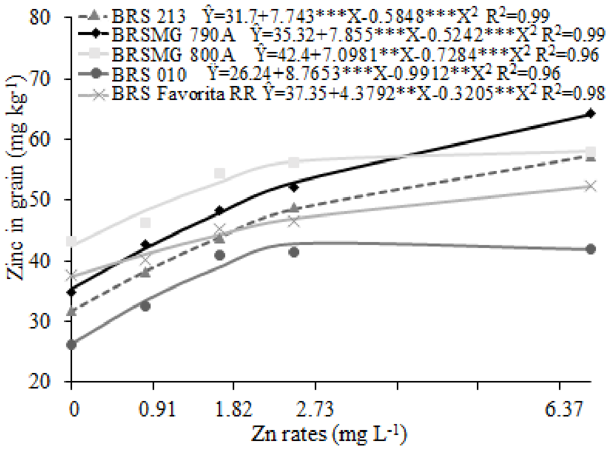

Figure 3- Zinc concentration in grains of soybean cultivars as a function of Zn rates fertilization via foliar.

The Zn contents found in this research are superior to the reported averages, evidencing the good accumulation of Zn in the grains when the fertilization is done by foliar applications. Zn values in soybean grains between 22 and 45 mg kg^{-1} are reported in the literature (Brunini *et al*., 2016; Esteves *et al*., 2010; Rigo *et al*., 2015).

The use of Zn rates resulted in efficiency of 39.87 to 81.44% of a Zn grain, with an increase of approximately 29 mg kg^{-1} between treatments 0 and 6.37 mg L^{-1} (Table 2). The highest increase in Zn content in the grain was found in the cultivar BRSMG 790A with 28.77 mg kg^{-1} more with the use of 6.37 mg L^{-1} compared to the control and efficiency of 81.44%. The BRS Favorita RR® presented the smallest increment (14.89 mg kg^{-1}) with an efficiency of 39.87%. The BRS 010 cultivar presented the lowest Zn content when compared to the other evaluated cultivars. These results show the difference between cultivars and the capacity of Zn accumulation in the grain.

Table 2- Estimated values of Zn concentration in the grain, using the rate 0 and 6.37 mg L^{-1}, increments and efficiency (%) and increase rate in food-type soybean cultivars. Lavras, MG, Brazil.

	Zn rates (mg L^{-1})			
Cultivar	0	6,37	INC[1/]	EF[2/]
BRS 010	26.24	42.78*	16.54	59.51
BRS 213	31.70	57.29	25.59	80.74
BRSMG 790A	35.32	64.09	28.77	81.44
BRSMG 800A	42.40	58.06	15.66	36.93
BRS Favorita RR®	37.35	52.24	14.89	39.87

[1/]Increment = variable in Zn rate 6.37 mg L^{-1} – variable in Zn rate 0 mg L^{-1}, mg kg^{-1}; [2/]Eficiency (%)={[(variable in Zn rate 6.37 mg L^{-1} x100)/(variable in Zn rate 0 mg L^{-1})]-100};*value found with rate 2.73 mg L^{-1}

The results of mineral composition of soybean grains on dry basis are presented in table 3. No influence of the Zn rates on other nutrients accumulation in the soybean grains was verified, which shows that there was no competition between micronutrients transport of the leaves towards the grain when the foliar application was made in R4 grown stage. Significant differences were found only among the cultivars. For the macronutrients Ca, Mg and P the highest levels were presented by cultivar BRSMG 800A with 3422.83, 2242.841 and 4011.47 mg kg^{-1}, respectively. As for nutrient K, the highest content was found in cultivar BRSMG 790A with 13806.49 mg kg^{-1}, while cultivars BRS 010 and BRS 213 accumulated higher levels of S.

In relation to the micronutrient contents, cultivar BRS 213 showed higher levels of nutrients B, Cu, Fe and Mn. As for Mo, the highest content was obtained by the cultivar BRS 010. It is observed that the increase of Zn doses did not influence the Fe content, an important nutrient as the Zn for biofortification, although these nutrients present an antagonistic relation to Absorption and transport in plants. According to Moosavi and Ronagh (2001) the foliar application of nutrients that have antagonistic relationships such as Fe and Mn, are an effective and economical option to avoid yield loss and nutritional imbalance in Fe-efficient soybean plants grown in limestone soils.

Table 3- Means of lipid content and mineral composition of food-type soybean cultivars. Lavras, MG, Brazil.

	BRS 010	BRS 213	BRSMG 790A	BRSMG 800A	BRS Favorita RR®	CV(%)
	Lipid (% DM)					
	20.10 a	18.89 b	18.83 b	19.32 b	19.08 b	5.51
	Mineral composition (mg kg^{-1})					
Ca	2491.05 c	2720.05 b	1967.60 e	3422.83 a	2211.51 d	5.70
K	7867.30 b	8357.88 b	13806.49 a	8692.09 b	6981.92 c	14.43
Mg	1869.63 c	1986.09 b	1622.96 d	2242.84 a	1637.36 d	3.14
P	3.354.21 c	3.521.00 b	3.457.99 b	4.011.47 a	3.222.51 d	2.65
S	2.148.52 a	2.180.39 a	1.820.25 c	1.918.47 b	1.678.29 d	3.81
B	4.54 b	6.49 a	4.60 b	3.37 c	2.70 d	10.20
Cu	4.00 a	4.22 a	3.50 b	4.07 a	3.97 a	9.03
Fe	64.23 b	67.34 a	59.70 c	61.16 c	57.99 c	5.84
Mn	44.39 b	50.65 a	34.48 c	41.20 b	34.65 c	10.67
Mo	8.87 a	5.73 b	6.05 b	4.48 c	5.27 b	22.68

[1] Means followed by the same lower case letter in the line are from the same group according to Scott-Knott test ($p \leq 0.05$).

Although the success of agronomic biofortification, is necessary to synchronize the Zn foliar application together with other agronomic intervention already practiced in the routine of the production area. Research indicates that there is no antagonistic effect between insecticides and Zn when applied simultaneously contributing both to improve production and the concentration of Zn in the grains (Wanga *et al.*, 2015).

To effectively improve the human nutritional status, it is essential that nutrient in biofortified staple foods are bioavailable (Trijatmiko *et al.*, 2016). In case of greater bioavailability of Zn in the grain derived from the foliar application than via soil, it would make agronomic biofortification a very attractive, useful and effective strategy for solving global health problems (Cakmak, 2008). Althought, it is necessary to monitor the use of these techniques through technical and legal devices and studies that prove their need, in a safe way to the populations at risk, by periodically assessing nutritional status to ensure that the micronutrient is available and consumed by the population And the constant monitoring in the intervention programs (Vellozo and Fisberg, 2010).

CONCLUSION

The foliar fertilization with Zn rates increase plant height, grain mass and protein content in soybean grains. The use of the 6.37 mg L^{-1} applied via foliar in R4 stage promoted greater concentrations of Zn in soybean grains. The food-type cultivar BRSMG 790A showed the highest Zn content in the grains with the greatest efficiency of Zn use. The mineral composition of the grains was not affected by the use of foliar fertilization with Zn, except Zn. Therefore, these results will contribute to defining the utility and application of Zn to promote good increment of Zn in edible plant parts when the micronutrient is applied via foliar to biofortification specially when the soil content of the nutrient are low.

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ARTIGO 3 - ZN-INDUCED TOXICITY IS COUNTERACTED BY FE IN SOYBEANS

(VERSÃO PRELIMINAR)

Artigo elaborado segundo as normas da revista Australian Journal of Crop Science

Abstract

Many unknowns remain in the field of metal homeostasis in plants. Therefore, in the present work the effect of the Zn and Fe levels on soybean plants were examined. Soybean Seeds of BRS 290A genotype, indicated to human alimentation (food-type), were placed and grown in a hydroponic system containing modified nutrient solution. In the first experiment, the experimental design was a completely randomized factorial 4 x 3: four Zn levels (2, 10, 20 and 40 μM) and three Fe levels (1, 7.7 and 77 μmol L^{-1}) with four replicates. In the second experiment, plants were grown in modified uptake solution containing three Fe levels (1, 7.7 and 77.7 μmol L^{-1}) combined with high Zn concentration (40 μM) containing ^{64}ZnO. Plants were harvested at specific time points during 48 hours (1, 2, 4, 6, 10, 16, 22, 24, 32 and 48 hours later) of treatments. Were analysed the plant growth, visual diagnosis, Zn and Fe concentration in leaves, stems and roots, Zn and iron uptake and expression of genes involved in Zn/Fe uptake and transport. Different Zn and Fe levels combined affected the uptake and mineral status, as well as soybean plant growth promoting different symptoms of Zn toxicity when combined to low Fe levels.

Keywords: heavy metal; mineral nutrition; nutrient uptake; phytotoxicity.

Introduction

Zinc and Iron are essential nutrients for all living organisms. Biofortification to increase the mineral content of plants has been concentrated primarily on these nutrients, which are most frequently deficient in human diets (Hirschi, 2009). Zinc is the second most abundant trace element, followed by iron. This nutrient is a necessary cofactor for many biological reactions, integral component of proteins, catalytic factor and signaling mediator, limiting oxidative degradation of auxin and maintaining membrane integrity (Tsonev and Lidon, 2012; Kambe et al., 2015; Joy et al., 2015).

Micronutrients interactions affect their uptake, distribution, and utilization in plants (Imtiaz et al., 2003; Fageria, 2011). Some factors have influence in these interactions, such as concentration of nutrient, temperature, light intensity, soil aeration, soil moisture, soil pH, architecture of root, the rate of plant transpiration and respiration, plant age and growth rate, plant species and internal nutrient concentration of plants (Fageria, 2011). Several antagonistic effects between micronutrients were observed (Moosavi and Ronaghi, Kobraee et al., 2013; 2011). For example, Zn interferes in Fe uptake and translocation, while Fe interferes in Zn translocation, but only when Zn concentrations were high (Ai-Qing et al., 2011).

Heavy metal such as Zn and Fe also are considered toxic to plants, particularly when acquired in excess quantities (Shanmugam et al., 2011). Zinc toxicity promotes the inhibition of growth and decreases in biomass production; severe toxicity can also be fatal. Zinc toxicity is almost certainly involved with metabolism through competition for uptake, inactivation of enzymes, displacement of essential elements from functional sites (Rout and Das, 2003).

High levels of Zn induce Fe deficiency (Lesková et al., 2017). Moreover, plants under this situation reduce dry matter yield and show chlorosis, but this visual symptom is more expressive in plants grown under Fe deficient conditions than under Zn toxicity. This suggests that Fe deficiency is one of the main factors impairing the growth of plants exposed to Zn toxicity (Fontes and Cox, 1998). The potential toxicity of many minerals presents in soil, plants tightly regulate the processes of their imbibition and storage (Zielin´ska-Dawidziak and Siger, 2012).

The presence of Zn in high levels inhibits the Fe metabolism, promoting the appearance of symptoms of induced Fe deficiency. The typical symptom is a consequence of chlorophyll concentration decrease, showing chlorotic leaves which later become white and causing accentuated delay in plant growth. Fe has numerous physiological functions in plants, but, for the expression of visual symptoms, its involvement in chloroplast formation and porphyrin synthesis are the most important processes (Romheld, 2001).

The transport of zinc from the root to the shoot and further to the developing seed results from an interplay not only between different membrane transporters but also between the mobility of zinc both intracellularly and between organs by binding to ligands (Olsen and Palmgren, 2014). The understanding of these mechanisms might eventually allow a focused strategy for improving the Zn content of cereal grain via the enhancement of Zn uptake, transport and remobilization (Palmgren et al., 2008).

Zn uptake is influenced by other divalent cations such as Fe. This interaction occurs as a consequence of the competition with Zn for the same metal transporters and non selective cation channels, that can transport many divalent cations like Fe^{2+} in addition to Zn^{2+}. In this case, both metal ions have common transporter proteins required for their absorption (Gupta et al., 2016).

Several transcriptional regulators might be involved in the balanced Zn and Fe uptake from the transporters. The involvement of chelator complexes and their roles in facilitating the control of metal uptake and tolerance are not known. More research in these directions is needed (Shanmugam et al., 2013).

The goal of biofortification is to develop plants that have increased content of bioavailable nutrients in their edible parts, like seeds (Stein et al., 2008). The quantities of minerals in seeds depend on uptake from the rhizosphere into the roots, translocation to the transpiring shoots in the xylem, transferring into leaves or other tissues, and finally, translocation into the seeds in the phloem. A major challenge of biofortification is an incomplete understanding of the pathways and the rate limiting steps involved in translocating minerals to the seeds (Waters and Sankara, 2011).

Soybean [*Glycine max* (L.) Merrill.] is one of the most widely grown legume crops in the world (Ramamurthy et al., 2014). In soybean, zinc interferes with the translocation of iron by inhibiting the reducing capacity of the root (Fe^{3+} - Fe^{2+}) or accentuating other reactions detrimental to iron transport (Ambler et al., 1970). A comprehensive understanding of the genetics underlying overall seed nutritional composition is lacking in soybean (Ramamurthy et al., 2014).

Given the importance of metals to the survival and proper function of plants, and given the importance of plants to nutrition and energy, it is imperative that research address the many unknowns that remain in the field of metal homeostasis (Palmer and Guerinot, 2009). Therefore, in the present work the effect of the Zn and Fe levels on plant growth, total Zn and

Fe content and the expression of genes involved in Zn/Fe uptake and assimilation were examined.

Materials and Methods

Plant material and growth conditions

Soybean Seeds of BRS 290A genotype, indicated to human alimentation (food-type), were disinfected in 10% NaOCl solution for 10 minutes, rinsed with distilled water, and germinated on moistened filter papers roll in room temperature (±22°C) for 6 days. After that, uniformed seedlings were selected and placed in 2.2 L dark pots (experiment 1) and 30 L dark box (experiment 2) in a hydroponic system containing modified Magnavacca nutrient solution (Magnavaca et al., 1987). The nutrient solution contained 3.25 mM Ca (NO_3) $2.4H_2O$, 1.3 mM NH_4NO_3, 0.57 mM KCL, 1.16 mM K_2SO_4, 0.57 mM KNO_3, 0.85 mM $Mg(NO_3)2.6H_2O$, 0.043 mM KH_2PO_4, 77µM $Fe(NO_3)3.9H_2O$,75 µM HEDTA, 9.1 µM $MnCl_2.4H_2O$, 25 µM H_3BO_3, 2,29 µM $ZnSO_4.7H_20$, 0.63 µM $CuSO_4.5H_20$, and 0.83 µM $Na_2.MoO_4.2H_20$. Plants were grown in a greenhouse at USDA Department from Cornell University, Ithaca, New York. The plants was kept at day temperature of 30°C (±2) and 25°C (±2) at night, with 12 h photoperiod. The solution was maintained in constant volume, aeration and pH at 5.5 (adjusted by 0.1 mol L^{-1} NaOH or HCl when necessary).

Mineral treatments

For the treatments of young plants growing hydroponically in 2.2 L dark pots, the experimental design was a completely randomized factorial 4 x 3: four Zn $(ZnSO_4.7H_2O)$ levels (2, 10, 20 and 40 µM) and three Fe $(Fe(NO_3)3.9H_2O$ + HEDTA) levels (1, 7.7 and 77 µmol L^{-1}) with four replicates in total 58 pots. Each experimental unit was made up of four plants per pot. After 15 days of treatments, plants were harvest individually into shoots and roots, rinsed using distilled water and 10 mM $CaCl_2$, and either dried at 65°C for three days for mineral analysis or frozen at -80° C for other analyses.

Visual diagnosis and Growth parameters

The 15-d-old plants were observed to see some different symptoms comparing with plants that growing in normal nutrient concentration. Measurements of growth parameters, including shoots and roots fresh weight (g), plant height and root length (cm) were made.

Analysis of mineral contents

Dried shoots and roots were grounded into powder. Approximately 200 mg powdered samples were weighed into borosilicate glass tubes, and acid-digested with 3.0 mL of $HNO_3/HClO_4$ (60/40%, v/v) at room temperature overnight. After that, samples were heated to 120° C for two hours. The mixtures were then heated to 120° C for two hours and 0.25 ml of 40 ppm Yttrium added as an internal standard to compensate any drift during run in Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES). The temperature of the heating block was then raised to 145°C for two hours. When necessary, more nitric acid (1-2 ml) was added to destroy the brownish color of organic matter. Then the temperature of the heating block was raised to 190° C for ten minutes and turned off. The cooled samples in the tubes were diluted to 20 ml, vortexed and transferred into auto sampler tubes to run in ICP. The model of the ICP used was Thermo iCAP 6500 series.

RNA preparation and qRT-PCR

Total RNA was extracted from very young leaf and root (15-day-old) samples using Trizol reagent following the manufacturer’s instruction (Invitrogen, Carlsbad, CA). RNA (1 μg) was primed with oligo (dT) and reverse-transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). The synthesized cDNA was diluted 5 times in water and the quality was checked based on amplification with *actin* gene primers. Quantitative RT-PCR (qRT-PCR) was done using gene-specific primers (Table 1) following the method as described (Zhou et al., 2011). The analysis of all gene expression was run in duplicate with three biological repeats.

Table 1 Primers used for quantitative real-time reverse transcription PCR

Target Gene	Primer name	Forward primer (5’-3’)	Reverse primer(5’-3’)
ACTIN 11	Glyma18g52780	AGAACTGGAGACAGCCAGGA	CTGGACATCTGAAACGCTCA
HMA2	Glyma 09g055600	AACTGGGGAACTTGTTGACG	CACTTTCCTTCCACCACGAT
IRT1	Glyma 07g34930	GGTGGTCTTTTTCTCCGTCA	AGCAAACCCACGGTGATTAG
MTP1	Glyma 16g017800	TTCCTGATGGTGGAAGGAAG	CAAAGGCTGCAACATCTGAA
ZIP1	Glyma 20g063100	GCTTTCGAGAACCTCACGTC	TCATGCCCTGATTCCTTTTC
ZIP4	Glyma 06g052000	GGCAACATCAAGGAACAGGT	ACAATCCCAAGCTCCAACAC
ZIP5	Glyma 19g049100	GCCTCTTTCTAACGCACAGG	TGGCAATAAGGCCACCTATC

Uptake experiment

For the uptake experiments, the 7-d-old seedlings were transferred to a 30 L dark box containing the Magnavacca nutrient solution (without Fe) and kept for 24 hours. After that, plants were removed, immersed in deionized water for 2 min, and placed in modified uptake solution containing three Fe levels (1, 7.7 and 77.7 μmol L $^{-1}$) combined with high Zn concentration (40 μM) containing ^{64}ZnO. Plants were harvested at specific time points during 48 hours (1, 2, 4, 6, 10, 16, 22, 24, 32 and 48 hours later) of treatments.

The uptake experimental design was a completely randomized factorial 3 x10 (3 Fe levels and 10 time points). In each time point, four plants were harvest individually and roots were rinsed in the order of using distilled water, 10 mM $CaCl_2$ and distilled water. Plant samples were dried at 65°C for 3 days. Powdered samples of 200 mg were acid-digested and used for mineral analysis by ICP (Inductively coupled plasma, Agilent 7500GS module ICP-MS).

Data analysis

All results were analyzed using ANOVA to verify the significant differences between treatments using Test-F. Certain sets of data were also subjected to linear correlation and regression analysis, and also tested for their significance by measuring the correlation coefficient and determination Standard errors for all means were calculated.

Results and Discussion

Plant growth phenotype following zinc and iron treatments

The soybean plants were grown hydroponically in the nutrient solution containing increasing Zn levels from 2 µM (approximately normal Zn level in the nutrient solution) to 40 µM along with Fe at low (1 µM and 7.7 µM) and high (77.7 µM, normal Fe level in the nutrient solution) levels. The plants exhibited different growth phenotypes in response to various Zn/Fe treatments for 15 days. Consistent and visually discernable differences were observed (Figure 1).

Plants under treatments with 1 µM Fe combined with high Zn level (20 and 40 µM) or with 7.7 uM combined with 40 uM Zn showed typical symptoms of Fe deficiency and Zn toxicity, as indicated with interveinal chlorosis of the upper leaves along with some necrosis of tissue and apparent stunning of growth and foliar area (Fig. 1). While the low Fe conditions combined with high Zn levels showed chlorosis, the low and normal Fe levels combined with low Zn produced soybean leaves with dark intensity. The high Zn induced leaf chlorosis symptoms in low Fe levels was reduced when Fe levels were increased. The chlorosis may arise partly from an induced iron (Fe) deficiency as hydrated Zn^{+2} and Fe^{+2} ions have similar radii (Marschner, 2012).

The addition of 7.7 uM Fe instead of 1 uM decreased the leaf chlorosis symptoms in plants treated with 20 uM Zn, while 77.7 uM Fe rescued the leaf chlorosis phenotype in the presence of 40 uM Zn (Fig. 1). The presence of Zn in excess can induce Fe deficiency in plants and show phytotoxicity symptoms (Chaney, 1993). However, the presence of Fe in high concentration promoted greater tolerance of the plants to the symptoms of phytotoxicity caused by high concentrations of zinc.

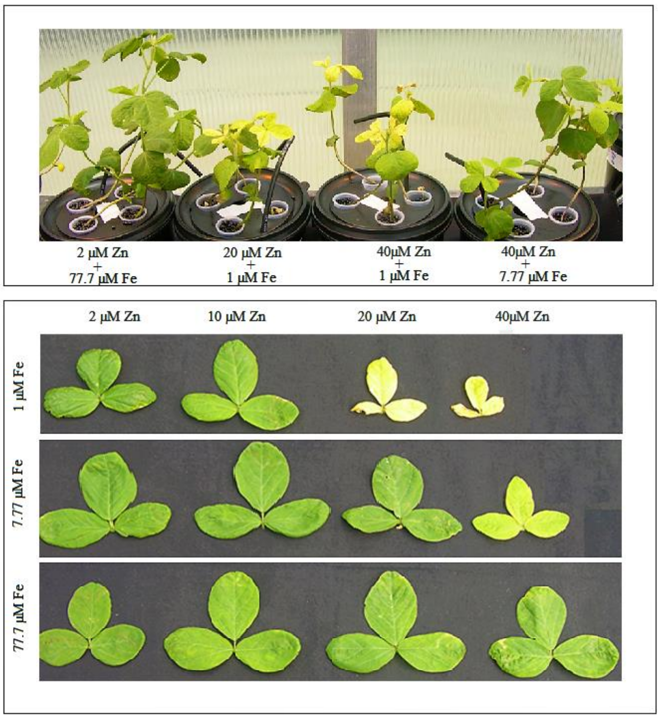

Figure 1 Visual appearance of leaves of soybean seedlings after exposure to different Zn and Fe treatments for 15 days of growth.

Plant grown parameters were significantly affected by Zn and Fe treatment levels (Table 1). In the presence of low Fe dose (1 µM), plant height and plant weight were dramatically reduced with increasing levels of Zn. Interestingly, plant root length and weight were increased. In the presence of normal Fe dose (77.7 µM), plants grew well with the highest height average (21 cm) when combined with Zn dose of 2.29 µM. Low Fe combined with high Zn concentrations resulted significant decrease in plant height and weight. Seedlings that received above 20 µM of Zn presented smaller plant height and weight when compared with normal Zn concentration (2.29 µM). High levels of Zn in soil are known to inhibit many plant metabolic functions, result in retarded growth and cause senescence (Nagajyoti et al., 2010).

Table 1 Plant height, root length, plant and root weight induced by Fe and Zn levels

Zn dosage (µM)	Fe dosage (µM)		
	1	7.7	77.7
	Plant Height (cm)[a]		
2.29	18.00 ± 1.7	18.66 ± 1.1	21.00 ± 3.4
10	14.33 ± 0.5	17.66 ± 1.1	19.33 ± 0.5
20	13.00 ± 1.7	18.00 ± 0.0	18.33 ± 0.5
40	13.33 ± 0.5	14.66 ± 1.1	18.66 ± 0.5
ANOVA[b]	Zn treatment	****	
	Fe treatment	****	
	Zn x Fe	NS	
	CV (%) =	8.22	
	Root lenght (cm)[a]		
2.29	39.00 ± 1.7	44.00 ± 1.7	43.33 ± 4.9
10	36.33 ± 3.2	46.33 ± 1.1	43.33 ± 1.1
20	50.66 ± 1.1	50.33 ± 4.5	52.00 ± 7.0
40	49.33 ± 0.5	44.00 ± 2.6	54.33 ± 2.30
ANOVA[b]	Zn treatment	*****	
	Fe treatment	*	
	Zn x Fe	NS	
	CV (%) =	7.05	
	Plant weight (g) [a]		
2,29	4.86 ± 0.2	5.43 ± 0.3	4.30 ± 0.1
10	4.36 ± 0.3	6.60 ± 0.6	5.70 ± 0.3
20	3.73 ± 0.3	5.76 ± 0.3	5.63 ± 1.0
40	3.63 ± 0.4	5.43 ± 0.1	5.56 ± 0.1
ANOVA[b]	Zn treatment	*	
	Fe treatment	****	
	Zn x Fe	***	
	CV (%) =	8.96	
	Root weight (g)[a]		
2,29	2.13 ± 0.2	2.66 ± 0.2	1.73 ± 0.3
10	1.90 ± 0.1	3.53 ± 0.3	2.53 ± 0.3
20	1.73 ± 0.3	3.13 ± 0.2	1.56 ± 0.2
40	2.16 ± 0.3	3.46 ± 0.2	2.73 ± 0.0
ANOVA[b]	Zn treatment	****	
	Fe treatment	****	
	Zn x Fe	NS	
	CV (%) =	11.44	

[a]Values are averages of three replicates ± SD (standard deviation). [b]ANOVA was performed and [NS] and ****, ***, **, * indicate non-significance difference at $p<0.0001$, 0.001, 0.01 and 0.05, respectively.

Zn and Fe accumulation in leaves, stems and roots

Zn and Fe contents in soybean plants treated with Zn and Fe were analyzed by ICP. The results of the statistical analyses showed that the total concentrations of Zn and Fe were statistically significant ($P<0.005$). As shown in Figure 2a, soybean seedlings exhibited difference in their leaf, root and stem Zn contents. The higher Zn accumulation in leaves, roots and stem was in treatments of 1 and 7.7 µM Fe combined with 10, 20 and 40 µM Zn.

Leaves accumulated 391.70 µg of Zn when applied 1 µM Fe with 20 µM Zn. High accumulation was observed too in 1 µM Fe + 40 µM Zn and 7.7 µM Fe + 40 µM Fe with 286.04 and 377.88 µg of Zn, respectively. Higher Zn accumulations in roots and stem were observed when concentration 7.7 µM Fe + 40 µM Zn was used (2488.41 µg of Zn and 1173.37 mg of Zn). These results is according with leaves symptoms obtained in this experiment.

Leaf and root Fe content increased with an increase in Fe concentration in the nutrient solution. In low Fe concentrations, 1 and 7.7 µM of Fe, the Fe content in leaves decreased with an increase concentrations of Zn supplementation. Differently, roots Fe content showed increase with both nutrient concentrations (Fe and Zn). A general Zn tolerance was observed in response to Fe increase.

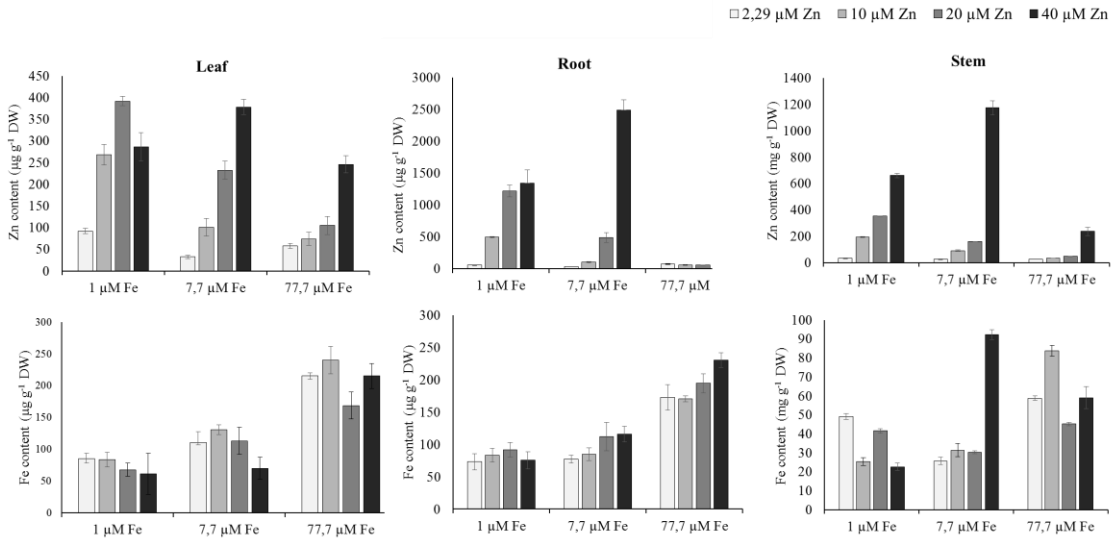

Figure 2 Zn and Fe concentration in soybean seedlings under Zn and Fe dosage. Error bars indicate standard deviation (±SD) (*n=3*).

Zinc toxicity in plants is clearly visible with the inhibition of growth and decrease in biomass production. Severe toxicity can also be fatal. It occurs because Zn is involved with metabolism through competition for uptake, inactivation of enzymes, displacement of essential elements from functional sites (Rout and Das, 2003).

In soybean, high concentration of Zn reduced the Fe concentrations to levels considered deficient, interfering with the crop development (Silva et al., 2014).

The Zn excess in A. thaliana can reduce the accumulation of Fe in shoots and thus induce significant Fe deficiency, and Fe excess could rescue the Zn stress even in plants with toxic levels of Zn in the shoot (Shanmugam et al., 2011).Additional mechanisms within the leaves exist to facilitate Fe and Zn transport between the vacuole and cytoplasm as part of a sequestration strategy, since high concentrations of either nutrient can be toxic for the plant cell (Pearce et al. 2014).

Zn and iron uptake under various Fe levels

To investigate the effects of Fe deficiency and sufficiency on Zn uptake in soybean seedlings, Zn and Fe uptakes were determined in plants exposed to 40 µM L^{-1} ^{64}ZnO under Fe deficient (1 and 7.7 µM L^{-1} Fe) and Fe sufficient (77.7 µM L^{-1} Fe) conditions for 48 hours in hydroponic system.

The interaction terms of those variables to test uptake in response to the high Zn with Fe level shows statistically significant difference. In this study, Zn and Fe influenced the uptake of each other. The best model was found and their estimates *P* values are in Figure 5. An increase in Zn uptake was observed in roots under Fe deficient conditions (Figure 5a). The Fe amount was significantly affected by Fe levels in high Zn concentration (40 µM L^{-1}). Higher Fe uptake was in the high Fe condition (Figure 5b). A Zn-Fe interaction had a clear mutually inhibitory action of Fe on Zn uptake by soybean plants.

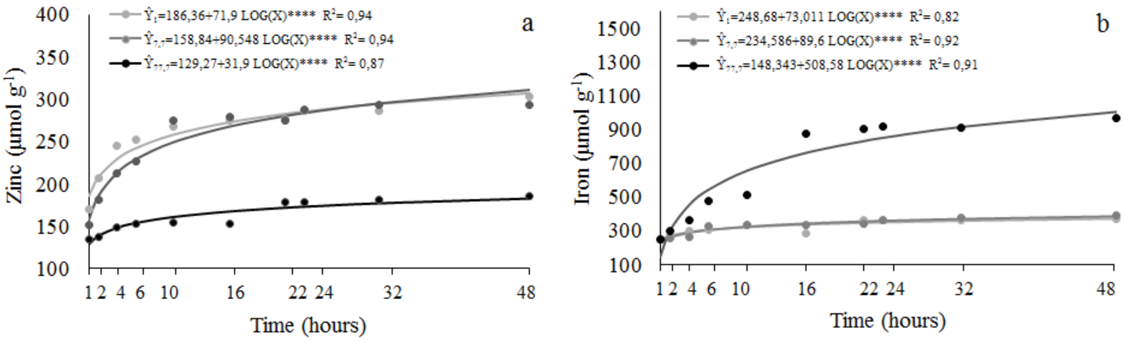

Figure 5 Zn and Fe concentration in soybean seedlings under high Zn and Fe deficient and sufficient conditions for 48 hours in hydroponic system.

In competition study using cells, was showed that Zn inhibited Fe uptake, and while Fe did not inhibited Zn uptake, when the metals were given together (1:1 ratio). These results point to a potential risk in the absorption and bioavailability of these minerals by the presence of other minerals in the diet. This aspect must be considered in food supplementation and fortification programs (Arredondo et al., 2006).

Due to the similar mechanisms of ions absorption, transportation and accumulation, the enormous Zn excess resulted in a selective suppression of absorption of other micronutrients in maize plants. The selectivity is related both to metal ion nature as well as to the plant organ. The more similar are these mechanisms, the higher are the suppression effects because Zn replaces other ions in the respective processes (Chilian et al., 2015).

Expression of genes involved in Zn/Fe uptake and transport

Several genes are involved in the Zn and Fe uptake and transport through the plant. Examination of genes expression encoding the key steps in the pathways should help to understanding of Zn and Fe homeostasis in soybean. In this study was investigated the transcript levels of some genes like *ZIP* family members, *HMA2* and *MTP1,* in soybean leaves and roots exposed to different Zn and Fe concentration levels in hydroponic system.

The proposed role of ZIP transporters in Zn nutrition has been further supported by characterization of homologs from several plant species (Ishimaru et al., 2011). Since *ZIP* is the key transporter for Zn and Fe uptake and translocation in plants, considerable progress has been achieved in cloning and characterizing its functions in crop plants, including soybean (Liu et al.,2013).

The ZIP transporter family plays roles in metal ion metabolism in a diverse array of eukaryotic organisms (Grotz et al., 1998). In this study, the *ZIP1* gene in leaves was expressed highly under low Fe and Zn condition (1 µM Fe and 2 µM Zn) (Figure 3a). In roots the expression decreased with increased Fe dosage when combined with high Zn dosage (Figure 3b).

The *ZIP* genes encode functional Zn or Fe transporters that may be responsible for the uptake, translocation, detoxification and storage of divalent metal ion in plant cells (Liu et al.,2013). According to Grotz et al. (1998) Zn was the most potent competitor, demonstrating that *ZIP1* prefers zinc as its substrate over other metal ions, Fe as example.

In soybean *ZIP1* encodes a symbiosis-specific zinc Transporter (Moreau et al., 2002).

The Zn deficiency increased expression of *ZIP4* in leaves and decreased according to the increase of Fe dosage in low Zn condition (Figure 3c). By the contrast, different expression of these gene was observed in roots, the high expression was found when plants were grown in high Zn condition (Figure 3d).

In rice, OsZIP4 encodes a Zn transporter localized to the plasma membrane and regulated by the plant Zn status. The induction of *OsZIP4* in Zn-deficient shoots occurred after the induction of *OsZIP4* in roots, and the expression of *OsZIP4* in shoots was gradually increased by prolonged Zn deficiency (Ishimaru et al., 2005).

Analysis revealed that OsZIP4 in Zn-deficient rice was expressed in the meristem of Zn-deficient roots and shoots, and also in vascular bundles of the roots and shoots, suggesting that OsZIP4 is a Zn transporter that may be responsible for Zn translocation to the plant parts that require Zn (Ishimaru et al., 2011).

As show in Figure 3 e-f, the gene *ZIP5* in leaves was higher in plants exposed to high Zn concentration when compared with low Zn, just when combined with low Fe (1 µM and 7.7 µM). Although, was higher expressed in plants exposed to high Fe (77.7 µM) combined with low Zn (2 µM). In roots, the higher expression was in plants under high Fe and Zn dosage.

Several members of the Zn-regulated transporters in the Fe-regulated transporter like protein (ZIP) gene family have been characterized and shown to be involved in metal uptake and transport in plants (Connolly et al. 2002)

ZIP1 is expressed in roots in response to Zn deficiency, suggesting that it transports Zn from the soil to the plant, while ZIP4 is expressed in both roots and shoots, suggesting that it transports Zn intracellularly or between plant tissues (Guerinot, 2000).

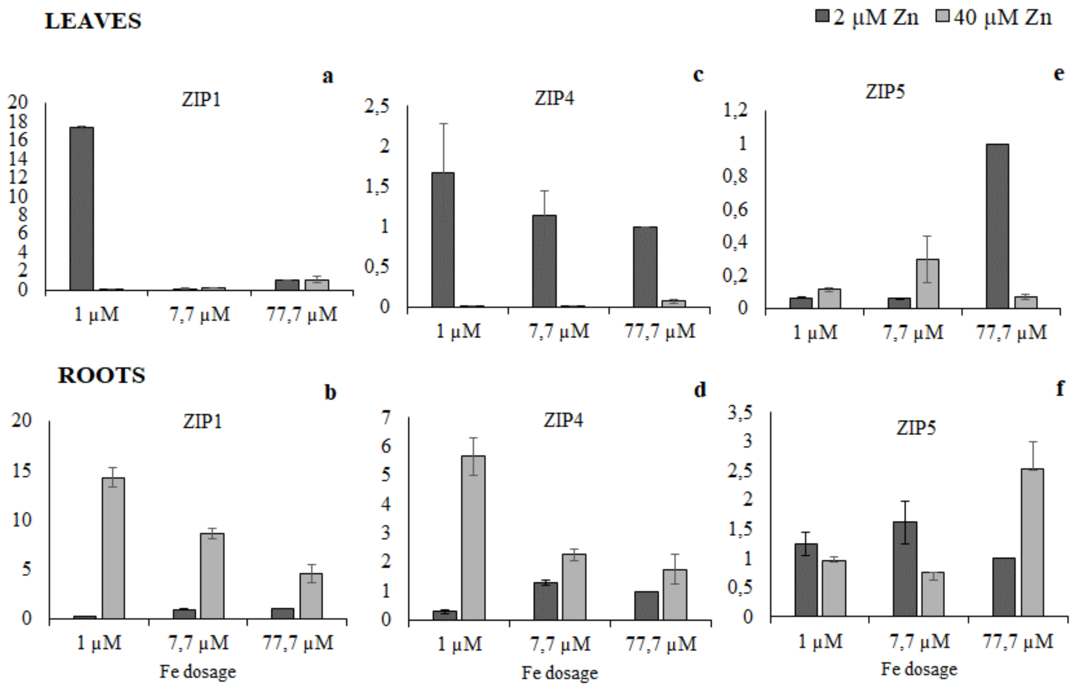

Figure 3 Relative gene expression of genes *ZIP1*, *ZIP4* and *ZIP5* involved in Zn and Fe uptake and transport inn soybean leaves and roots. Data are means of three technical trials with two biological repeats.

The *HMA2* is a member of the Heavy Metal ATPase (HMA) family of transporters. The majority soybean *HMA* genes are expressed in multiple tissues like roots, stems and leaves, suggesting that they have a general role in plant development (Hussain et al., 2004; Fang et al., 2016). A small number of these genes have tissue-preferential expression, suggesting that those genes may have specific roles in a certain cell context (Fang et al., 2016).

As show in figure 4b, *HMA2* gene was expressed at high levels in leaves and roots of plants under low Fe and low Zn solution concentration (7.7 µM L^{-1} of Fe and 2 µM L^{-1} of Zn). *HMA2* is localized to the plasma membrane and is involved in the translocation of Zn to various tissues of the plant and in phloem tissue, indicating a role in the remobilization of Zn from shoot to root, under conditions of both visible Zn deficiency and Zn supplementation (Hussain et al., 2004).

IRT1 is a plasma membrane transporter, involved in Fe homeostasis in plants and is the major root Fe uptake system in soil. Although, this gene mediates accumulation of other divalent metal cations to replace Fe in some cellular processes under low-iron conditions, Zn as example (Vert et al., 2002).

In this study, the *IRT1* showed increased expression in leaves with the increase of Fe in low Zn treatments (Figure 4c). The expression of *IRT1* is coordinately regulated at the level of transcript accumulation by Fe and Zn (Connolly et al, 2003).

In Arabidopsis this gene is the major high-affinity iron transporter, responsible for metal uptake from the soil solution under iron deficiency (Vert et al., 2002).

According to Shahzad et al. (2014) *OsMT1*a was found to be predominantly expressed in the roots and was induced by zinc treatment. The *MTP1* expression in leaves increased with the Fe dosage. The opposite was observed in roots, the higher expression was obtained with low Fe and Zn (1 µM L^{-1} of Fe + 2 µM L^{-1} of Zn).

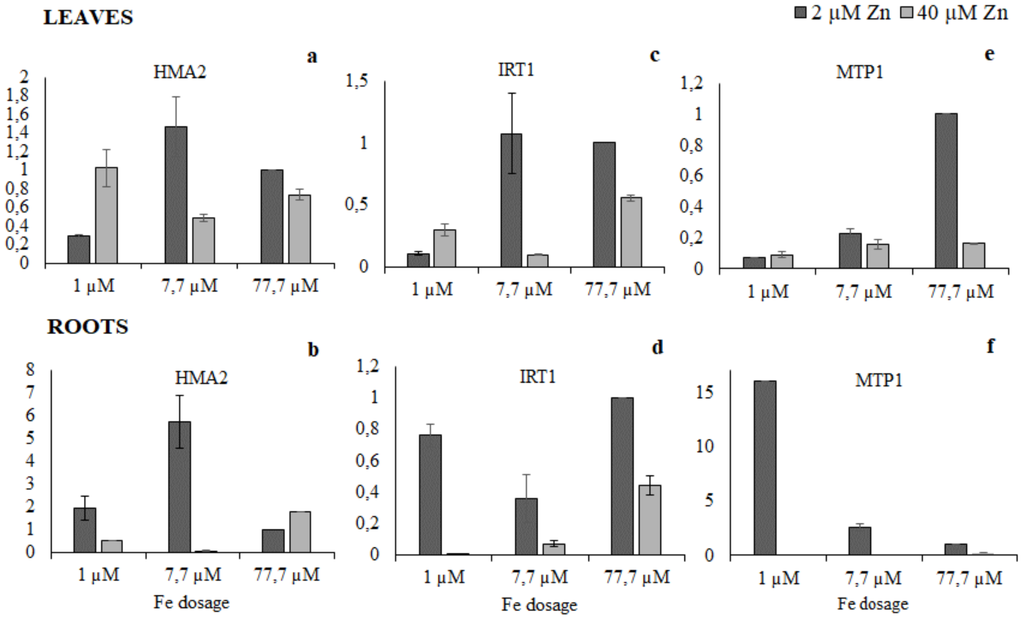

Figure 4 Relative gene expression of genes involved in Zn and Fe transport and assimilation, accumulation and tolerance in soybean leaves and roots. Data are means of three technical trials with two biological repeats

Enhanced accumulation of Fe and Zn in cereal grains can be achieved by fertilization or genetically manipulating of homeostasis-related genes. Proper functioning of cellular machinery requires a certain amount of Zn in the cytosol to serve the needs of cell organelles. But in excess of the nutritional needs, Zn might be transported to the vacuole to avoid cytotoxic effects which can be remobilized when required (Shahzad et al.,2014). Zn has cofactor role in many enzymes and is thought to play a role in signal-transduction and in gene regulation (Moreau et al., 2002)

Metals are transported from the soil into the root and then must cross both cellular and organellar membranes as they are distributed throughout the plant. Metal transporters may play different roles in this distribution process. Various molecular approaches ultimately can tell us not only in what tissue and cell type certain transporters are expressed but also where within a cell each is expressed (Guerinot, 2000).

Conclusion

In summary, different Zn and Fe levels combined affected the uptake and mineral status, as well as soybean plant growth promoting different visual symptoms of Zn toxicity when combined to low Fe levels. Stepwise analysis in our study indicated the Zn, was correlated with Fe concentrations in soybean. This study confirms that some genes are involved with Zn/Fe uptake and transport in soybean plants and plays a strong role in the regulation of these nutrients homeostasis. Zn and Fe level are involved in the upregulation of a large number of genes during the early stages of soybean. These genes appear to be interesting targets for plant breeding and biotechnological applications to improve nutrient uptake and transport.

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